UPPER RESPIRATORY TRACT DISEASE IN GOPHER TORTOISES, *GOPHERUS POLYPHEMUS*: PATHOLOGY, IMMUNE RESPONSES, TRANSMISSION, AND IMPLICATIONS FOR CONSERVATION AND MANAGEMENT

BY

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DEDICATION

This dissertation, the years that went into it, and the drive for knowledge that underpinned it, are a result of, and dedicated to, my parents:

Grace Slater Gibson "Billie" McLaughlin
1928-1978

and

Robert Douglas McLaughlin
1925-1994

Thank you.

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Upper respiratory tract disease (URTD) of tortoises is caused by the mollicute *Mycoplasma agassizii*, and is characterised by nasal and ocular discharge, palpebral edema, and conjunctivitis. Hyperplasia and dysplasia of the nasal passage and cavity epithelia and inflammatory infiltrates are seen histologically. In order to provide data for management decisions and to better understand URTD, I studied uninfected, naturally infected, and experimentally infected tortoises. The pathological and immune responses of tortoises to and transmission of *M. agassizii* were investigated using clinical and histological observations, culture and polymerase chain reaction (PCR) tests, and an enzyme-linked immunosorbent assay (ELISA). Infection with *M. agassizii* caused mild to severe damage to mucosal and olfactory nasal epithelia, with increased damage in longer-

term infections. Although clinically healthy, ELISA-positive, culture and PCR-negative tortoises may have eliminated the bacteria, when five such animals were examined at necropsy, three were found to harbor *M. agassizii* in the nasal cavities. When seropositive tortoises were challenged with *M. agassizii*, a more rapid and more severe clinical response resulted than on initial exposure, and plasma antibody levels began rising more quickly. When uninfected animals were housed with infected individuals, horizontal transmission occurred, probably via direct contact, but possibly via food, water, or fomites. Transmission was more likely to occur from a tortoise that was clinically ill and culture or PCR-positive. There was no discernible transmission when tortoises inhabited pens or entered burrows previously occupied by ill tortoises. There was no demonstrable vertical transmission, although there was transfer of maternal antibodies via egg yolk. The level of antibodies in egg yolk or hatchling plasma was approximately 10-20% of that in maternal plasma. Movement of tortoises during relocation, repatriation, or restocking efforts potentially could transport *M. agassizii* to previously uninfected sites. Because of the uncertainty involved in determining latent infections, ELISA-positive animals should not be moved to locations with no seropositive individuals. However, they can be used in captive breeding efforts and their offspring released into the wild. Clinically ill animals should not be relocated to new sites, and should not be housed with clinically healthy animals in temporary holding situations, such as on-site relocations, or in captive breeding programs.

CHAPTER 1
INTRODUCTION

Gopher Tortoise Natural History

Gopher tortoises, *Gopherus polyphemus*, are found in the southeastern United States, on the coastal plain from southern South Carolina south through Georgia and throughout Florida, and west through southern Alabama, Mississippi, and Louisiana. The major population concentrations are in Florida and southern Alabama and Georgia, with only remnant populations in South Carolina, Mississippi and Louisiana (Auffenberg and Franz 1982, Diemer 1992a). Populations are concentrated in areas with deep sandy soils suitable for digging. Vegetation associations in which tortoises are found include longleaf pine-xerophytic oak woodlands, palmetto scrub, sand pine scrub, oak scrub, beach scrub, coastal strands, pine flatwoods, dry prairies, native pasture, and savanna, as well as ruderal habitats (Landers and Speake 1980, Lohoefener and Lohmeier 1981, McRae et al. 1981, Campbell and Christman 1982, Diemer 1986, Breininger et al. 1988).

Gopher tortoises are an important element in the ecosystems in which they are found, and are considered by many ecologists to be a keystone species (Eisenberg 1983). Gopher tortoises live in loose colonies, with considerable movement of tortoises among groups over the years (Diemer 1992b). Colonies may be defined more by the availability of suitable soils for digging burrows, or the distribution of food resources, than by social

interactions (Campbell and Christman 1982). Gophers are the most fossorial of the four North American species of tortoises, digging burrows that may extend 5 meters down from the surface and 15 meters in length (Hansen 1963, Diemer 1986). The burrows provide a microclimatically stable environment for not only the tortoises, but also for numerous commensals. Approximately 60 vertebrate species--from snakes to birds--and over 300 invertebrates--including spiders, crickets, and beetles--have been found in tortoise burrows or observed using them as permanent homes or refuges from heat, cold, fire, and predators (Jackson and Milstrey 1989, Lips 1991, Witz et al. 1991). Some invertebrate species are obligate commensals, and occur only in active tortoise burrows, where they feed on tortoise feces and other invertebrates and, in turn, are eaten by other commensals, such as gopher frogs (*Rana areolata*) and Florida mice (*Podomys floridanus*) (Woodruff 1982). Several species that exclusively or frequently use tortoise burrows have legal protection in Florida and other parts of their ranges. These include scarab beetles (F. Scarabaeidae), indigo (*Drymarchon corais couperi*) and pine (*Pituophis melanoleucus*) snakes, gopher frogs, mole skinks (*Eumeces egregius*), burrowing owls (*Athene cunicularia floridana*), and Florida mice (Cox et al. 1987).

The soil disturbance resulting from the digging of the burrows allows deeper access of air and water into the soil profile, as well as providing bare mineral soil patches on the surface. When a burrow is abandoned the soil mound, or apron, in front of the entrance no longer undergoes continual disturbance, allowing certain plants to colonize the area. The composition of the plant assemblage on the mound may differ from that in the surrounding undisturbed area, providing a mosaic of small patches in the habitat (Breininger et al. 1988, McLaughlin 1990). Tortoises consume a wide variety of grasses

and forbes, and readily ingest the fruits of many shrubs when available (Garner and Landers 1981, Macdonald and Mushinsky 1988). Subsequent tortoise movements may help spread the seeds to suitable habitat, and some seeds (e.g., gopher apple) may germinate more readily after passage through the tortoise gut.

Gopher tortoises are long-lived, slow to mature, and have a low reproductive rate. Age estimates extend up to 100 yr, although 60-80 yr is considered a more reasonable estimate. Age to sexual maturity ranges from 10 to 20 yr, with possible latitudinal association (Alford 1980, Iverson 1980, Landers et al. 1982, Wright 1982, Doonan 1986, McLaughlin 1990, Mushinsky et al. 1994). Gopher tortoises lay one clutch of eggs annually, generally ranging from 2-10 eggs in size, with the average being 4-8 (Dietlein and Franz 1979, Iverson 1980, Landers et al. 1980, Linley and Mushinsky 1994). In some areas with excellent food resources and large tortoises, clutch sizes may average 9-10, with a range of 6-14 eggs (McLaughlin, 1990). Although some wild individuals have produced over 20 eggs in one clutch (Godley 1989, L. Macdonald, personal communication), this is very unusual. With predation rates of up to 95% on eggs and hatchlings, and further high losses of juveniles aged 1-5 yr, less than 10%, and possibly as low as 1%, of eggs laid eventually produce reproductive adults (Douglass and Winegarner 1977, Auffenberg and Iverson 1979, Alford 1980, Iverson 1980, Landers et al. 1982, Diemer 1986, Wilson 1991).

The gopher tortoise is listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (C.I.T.E.S.), which requires permits for the exportation of the species from the U. S. to any signatory nation, or for re-exportation (Levell 1995). However, if federal or state regulations are more restrictive

than C.I.T.E.S., those take precedence. Legal protection is extended to the species in all states within the range, although the levels of protection vary. The populations west of the Tombigbee and Mobile Rivers in Alabama, Mississippi and Louisiana are on the federal threatened species list, which prohibits the taking, exportation, or interstate movement of individuals originating from that region without permit, and the possession, transportation, purchase, and/or sale of illegally obtained specimens. Permits issued by the U. S. Department of Interior can be obtained for scientific research on wild populations, and possession of limited numbers of individuals for exhibition, education, and/or research. State permits are required also. The gopher tortoise is listed in Alabama as a protected nongame species, in Mississippi as endangered, and in Louisiana as threatened. Louisiana regulations also prohibit the use of gasoline, chemicals, or volatile substances to flush reptiles from burrows or other hiding places. Georgia lists the species as threatened and issues scientific collection permits only to qualified institutions and individuals for educational or research purposes. Although the law states that burrows may not be disturbed nor destroyed, nor may explosives, chemicals or smoke be introduced into them to drive out wildlife, the code explicitly exempts poisonous snakes. Because venomous snakes, particularly rattlesnakes, use gopher tortoise burrows, tortoises can be adversely impacted by such activities. South Carolina was the last state to extend legal protection to gopher tortoises (Mann 1990, Levell 1995), and now lists the species as endangered, with permits required for any activities involving tortoises. In Florida, where the gopher tortoise is listed as a species of special concern (Wood 1996), regulations prohibit taking and disturbing of tortoises and their habitats, although exceptions are granted regularly for development, agriculture, and mining operations. In the past, human consumption of

tortoises was a major impact on regional distribution, and current poaching activities may extirpate local aggregations (Diemer 1989, Mann 1990). Large scale conversion of long-leaf pine habitats to slash pine plantations, transformation of native pasture or savanna to "improved" pasture, citrus groves, or row crops, mining operations such as phosphate, mineral sand, and gravel mines, and urban/suburban development are the main threats to continued gopher tortoise survival today (Diemer 1986, Cox et al. 1987, Diemer and Moore 1994). Lack of natural fire regimes due to suppression efforts by humans may alter vegetation mosaics in remaining habitat, rendering them less suitable to continued maintenance of tortoise populations (Mushinsky 1986, Mushinsky and Gibson 1991).

Although tortoise numbers increase with increasing areal extent of available habitat, densities remain constant or decrease (Mushinsky and McCoy 1994). Fragmentation of mainland areas, with ever smaller islands of suitable habitat surrounded by agricultural and urban development, may force tortoises into higher density populations than would occur normally. Vegetational changes resulting from reduced fire incidence in small areas, particularly increased canopy closure and decreased herbaceous vegetation (Mushinsky 1985, 1986), can lead to decreases in reproductive rates and juvenile survival (Auffenberg and Franz 1982, McLaughlin 1990, Mushinsky and McCoy 1994). Increased intraspecific interactions at higher densities (McRae et al. 1981) may lead to elevated physiological stress, potentially affecting immune system function and rendering animals more susceptible to disease. Increased densities and number of interactions multiply the opportunities for transmission of communicable agents, making fragmented populations more likely to sustain high morbidity during epizootics. If mortality is high, lowered reproduction due to habitat degradation may be insufficient for population recovery.

Along with increased physiological stress placed on tortoise populations from human activities, there may be toxicological or immunological stress from chemicals introduced into the environment. No research has been conducted into the effects of herbicides, fungicides, insecticides, and fertilizers on gopher tortoise health, growth, or reproduction. Investigations of disease in free-ranging populations of tortoises have begun only recently.

Tortoises and Upper Respiratory Tract Disease

Although individual captive and wild gopher tortoises have been observed with clinical signs of respiratory diseases for over 20 yr (E. R. Jacobson, unpublished data), the first documentation of a larger-scale disease outbreak was in 1989, when an epizootic of upper respiratory tract disease (URTD) was documented on Sanibel Island, Lee County, Florida (G. S. McLaughlin and M. Elie, unpub. data). With the loss of 25-50% of breeding-age adults in one population, recovery could take 50-150 yr (G. S. McLaughlin unpub. data), barring further major losses and without substantial habitat improvement leading to increased recruitment.

In the 1980s, large-scale population reductions (33-76% over 10 yr) of desert tortoises (*Gopherus agassizii*) were documented at several sites in the western Mojave Desert of California and at one site in the eastern Mojave (Corn 1994, Berry in press). Tortoises with clinical signs of URTD were observed among the remaining populations at several sites (Knowles 1989; Berry 1990, in press). As a result of the declines, tortoises in the Mojave Desert north and west of the Colorado River were declared threatened (U.S. Fish and Wildlife Service 1990).

Clinical signs of URTD in gopher and desert tortoises include serous, mucoid, or purulent discharge from the nares, excessive tearing to purulent ocular discharge, conjunctivitis, and edema of the eyelids and ocular glands (Jacobson et al. 1991, G. S. McLaughlin personal observations). Individual infected tortoises vary in the suite of signs they have, and the severity can vary from day to day. Nares may become occluded with caseous exudate, preventing externally visible nasal discharge. Lymphocytic infiltration of the corneas, while rare, may decrease an animal's ability to forage or avoid predators. Tortoises may become lethargic and anorectic, leading to dehydration, emaciation, and eventual death from cachexia. Lethargy, nonresponsiveness to stimuli, and altered behavior patterns--such as basking at lower temperatures than normal--may render a tortoise more susceptible to predation. Moribund animals often develop petechial to ecchymotic hemorrhages under the scutes, especially visible on the plastron (G. S. McLaughlin pers. obs.), which may be due to septicemia caused by secondary infection with opportunistic bacteria.

Several agents were hypothesized to cause respiratory tract disease in tortoises, including viruses, *Mycoplasma* sp. (Lawrence and Needham 1985), and *Pasteurella testudinis* (Snipes and Biberstein 1982). However, experimental infections to determine the etiologic agent were not conducted. Beginning in 1989, efforts were undertaken to determine the etiology of URTD. Clinically ill and healthy desert tortoises from California were examined and samples collected, either from live animals or at necropsy. Hematologic and serum biochemical evaluations, liver vitamin and metal determinations, and pathologic and microbial investigations were conducted (Jacobson et al. 1991).

Hemoglobin concentration and phosphorous levels were lower in ill than in healthy tortoises, and ill tortoises had higher sodium, blood urea nitrogen (BUN), creatinine, serum glutamic oxalacetic transaminase (SGOT) activity, and total cholesterol levels. Levels of liver and serum vitamins A and E did not differ between healthy and ill tortoises, nor did liver values of selenium, copper, lead, or cadmium. However, iron and mercury were higher in ill than in healthy tortoises. Healthy tortoises did not exhibit any histological abnormalities in nasal mucosa or other tissues. Ill tortoises had less fat than controls, atrophied thymuses, increased numbers of lymphocytes in the sinusoids of the spleens, and increased amounts of iron granules in the hepatocytes. The most striking and consistent changes were in the nasal passage and cavity tissues (Jacobson et al. 1991). Grossly, passages and cavities of ill tortoises contained moderate to large amounts of serous to purulent exudate. Histologically, the tissues exhibited loss of mucosal glands and goblet cells, proliferation of epithelial cells, infiltrates of lymphocytes and histiocytes, loss of cilia, dysplasia of the olfactory epithelia with infiltration of heterophils, basal cell hyperplasia, occasional squamous metaplasia, and occasional erosion and ulceration of mucosal epithelia. By electron microscopy, pleomorphic organisms resembling *Mycoplasma* sp. were seen on cell surfaces and tightly adhered to cell membranes of ill tortoises, but not healthy ones. Significantly more *Pasteurella testudinis* were isolated from ill than healthy tortoises, and *Bacillus* sp. and *Mycoplasma*-like organisms were isolated only from ill tortoises. By electron microscopy, the latter resembled those seen on the nasal cavity tissues of ill tortoises (Jacobson et al. 1991).

The mycoplasma was determined to be a new species, provisionally named *Mycoplasma agassizii* (Brown et al. 1994). An enzyme-linked immunosorbent assay

(ELISA) to detect antibodies against the mycoplasma in plasma and serum samples was developed (Schumacher et al. 1993), and experiments were undertaken to fulfill Koch's postulates. The disease was induced by inoculation of tortoises with pure cultures of the mycoplasma, but not *Pasteurella testudinis* (Brown et al. 1994). Histologically, the lesions were consistent with those seen in the previously examined naturally infected tortoises. Additional work led to the development of a polymerase chain reaction (PCR) test to detect the bacteria in nasal flush and swab samples (Brown et al. 1995).

Histologic examination of nasal tissues and microbiologic evaluation of flush and swab samples from clinically healthy and clinically ill tortoises from Las Vegas Valley, Nevada, resulted in findings in the ill tortoises similar to those in the California tortoises, with 92% showing lesions of URTD, 50% being culture positive for *M. agassizii*, and 100% reacting positively in the ELISA (Jacobson et al. 1995). However, 73% of the "healthy" tortoises had lesions consistent with URTD, 50% were culture positive for *M. agassizii*, and 42% were seropositive for antibodies against the bacteria. These findings demonstrate that the disease can exist in a subclinical form in a substantial proportion of a population. An annual cycle of convalescence and recrudescence of clinical signs has been seen in captive desert (I. M. Schumacher, pers. comm.) and gopher (D. L. Morris, pers. comm.) tortoises. Other mycoplasmal diseases also can exist as chronic, subclinical infections, with recurrence of clinical signs and increases in transmission potential when the host is stressed (see below). Annual fluctuations in temperature, rainfall, and forage availability may be sufficient to cause detectable outbreaks in an infected population. Increased morbidity and mortality may occur in times of unusually severe environmental stress, such as prolonged drought, hurricanes, excessive rainfall with flooding of burrows,

or very cold winters. Human impacts on tortoises and their habitat, whether through disruption of normal behavior patterns, degradation of habitat through agriculture, silviculture, mining or development operations, or pollution, may cause sufficient physiological stress to trigger proliferation of the mycoplasma and recurrence of signs. Capturing and transporting of tortoises during relocation, restocking and repatriation efforts also may be significant sources of stress.

Mycoplasmal Respiratory Diseases in Domestic Animals and Humans

Mycoplasmas cause respiratory disease in other taxa, including rodents, poultry, swine, ruminants, and humans. All have similar pathological effects, which are described below, and many are exacerbated by concurrent infections or environmental stress.

Murine respiratory disease caused by *Mycoplasma pulmonis* has caused problems in laboratory settings for more than 70 yr (Lindsey et al. 1971). It has necessitated complex and expensive control measures, including various types of isolation mechanisms and cesarean delivery of mice and rat pups to reduce the prevalence of *M. pulmonis* infections in colonies (Cassell et al. 1984, Davis et al. 1987). Interactions among environment, host, and strain factors influence the impacts at individual and population levels (Simecka et al. 1992). Histologically, lesions are characterized by lymphoid hyperplasia and chronic inflammation (Cassell et al. 1985).

Poultry respiratory diseases can be caused by viruses, mycoplasmas, and other bacteria, singly or in combination. Without concurrent viral or other bacterial infections, disease can be mild or subclinical (Kerr and Olson 1967). Environmental factors, particularly dust and ammonia levels, as well as strain differences, affect the severity of

outbreaks of mycoplasmosis. Other stress factors, such as crowding and cold weather, also influence morbidity and mortality (Jordan 1972). *Mycoplasma gallisepticum* causes chronic respiratory disease in chickens and sinusitis in turkeys, and affects ring-necked pheasants (*Phasianus colchicus*), chukar (*Alectoris chukar*), northern bobwhite (*Colinus virginianus*), common peafowl (*Pavo cristatus*) (Yoder 1991) and Japanese quail (*Coturnix japonica*) (do Nascimento and do Nascimento 1986). Lower respiratory tract complications are seen rarely in turkeys (Simecka et al. 1992). Histologically, hypertrophy and hyperplasia of respiratory epithelia, including mucous cells, are seen, as is lymphoid infiltration of the submucosa. Heterophilic exudate is seen in the airways (Nunoya et al. 1987, Trampel and Fletcher 1981). *Mycoplasma meleagridis* infection is seen primarily in chicks and poults up to 10 wks of age, and is sexually transmitted. Turkeys, Japanese quail, and peafowl develop air sacculitis, sometimes with accompanying tracheitis and pneumonia, but not sinusitis. Histologically, the lesions are characterized by hypertrophy and hyperplasia of the air sac epithelia, with edema and lymphoid infiltration (Stipkovits 1979). Young chickens and turkeys also are susceptible to *M. synoviae*, usually in conjunction with Newcastle disease virus or infectious bronchitis (Hopkins and Yoder 1984, Springer et al. 1974). As with *M. gallisepticum*, turkeys develop sinusitis (Stipkovits 1979). All three bacteria can be transmitted vertically (i.e., via the egg) (Simecka et al. 1992).

Swine develop mild pneumonia when infected by *M. hyopneumoniae,* and although mortality is virtually nil, the disease is chronic, causing slow growth and reduced weight gains, decreasing profitability (Oboegbulem 1981, Jericho 1986). Although signs can disappear, recurrences will occur with weather changes, viral infections, and other

stressors (Whittlestone 1976). Lesions are alveolar, initially characterized by neutrophilic infiltrates and later by lymphocytes and macrophages (Baskerville 1972).

Contagious bovine pleuropneumonia (CBPP), although not present in the United States, is an economically important disease of cattle and water buffalo caused by *M. mycoides* subsp. *mycoides* (Howard and Gourlay 1978, Trichard et al. 1989). Morbidity rates can reach 100% with up to 50% mortality, and recovered animals maintain a carrier state. Neutrophilic exudate in the airways, serous exudate in the pleural cavity, serofibrinous exudate in alveoli, and edema and necrosis of regional lymph nodes and interlobular septa are seen at necropsy. Interseptal, peribronchial, and perivascular lymphoid infiltration can be seen histologically (Hudson 1971). Calf pneumonia, with high mortality among dairy calves, is caused primarily by viruses, but can be caused by either *M. bovis* or *M. dispar* (Stalheim 1983, Bryson 1985). Infection with the former is characterized by peribronchiolar and alveolar monocyte infiltration, while the latter causes interstitial pneumonia with monocyte infiltration of alveolar walls, but not peribronchiolar infiltration. *Mycoplasma dispar* also causes superficial and asymptomatic infection of respiratory mucosa (Woldehiwet et al. 1990).

Lambs are susceptible to infection with *M. ovipneumoniae*, which is transmitted to neonates from the ewes. Pneumonia, characterized by coughing, sneezing, nasal discharge, fatigue, and poor weight gain, can develop as colostral antibodies wane. Lesions are characterized by alveolar proliferation, nodular lymphoid hyperplasia, and peribronchial and perivascular lymphoid infiltration (Carmichael et al. 1972, Foggie et al. 1976). Severity can be exasperated by infection with *Pasteurella haemolytica* biotype A (Jones et al. 1982).

Goats can suffer 60-100% mortality when infected by *M. mycoides* subsp. *mycoides, M. mycoides capri*, or *Mycoplasma* strain F38, making contagious caprine pleuropneumonia the most economically important goat disease. Pathologically, signs and lesions are similar to those seen in CBPP, with the addition of polyarthritis (Cottew 1984).

Humans are susceptible to infection with *M. pneumoniae*, which causes tracheobronchitis and, less commonly, primary atypical, or walking, pneumonia (Chanock et al. 1963, Clyde 1983). The upper respiratory tract is affected, not the alveoli, and otitis media is seen also (Clyde 1979, Mansel et al. 1989). Immunopathological sequelae, although rare, can include arthritis, dermal lesions, cardiopathy, and neurological complications (Johnston et al. 1983, Lyell et al. 1967, Naftalin et al. 1974, Murray et al. 1975). Histologically, perivascular and peribronchial monocytic infiltrations with some neutrophilic exudate are seen (Dajani et al. 1965). Most commonly affected are 5-9 yr old children, with a decline in incidence until about age 25, then an increase in 30-40 yr olds (Denny et al. 1971). The disease is endemic, with slight increases in late summer and fall, and cyclic epidemics occurring at 4-7 yr intervals (Krause and Taylor-Robinson 1992).

Mycoplasmal Diseases of Wildlife

Research on the impact of mycoplasmosis on wildlife is limited, but recent developments are provoking interest. In early 1994, house finches (*Carpodacus mexicanus*) in the mid-Atlantic and northeastern regions of the United States were found with conjunctivitis, rhinitis, and sinusitis. Many birds were found dead or dying and were submitted to wildlife care facilities, veterinary hospitals, and state agricultural laboratories. Many of those birds had lesions that were histologically compatible with mycoplasma

infections, and were infected with *M. gallisepticum* (Ley et al. 1996, Luttrell et al. 1996). Since then, the organism has been associated with morbidity and mortality in other species of passerines, including goldfinches (*Carduelis tristis*) (Nettles 1996), and was transmitted to a blue jay (*Cyannocitta cristata*) in a rehabilitation facility (Ley et al. 1996). Conjunctivitis and *M. gallisepticum* also were found in house finches in Georgia (Luttrell et al. 1996). The potential for this organism to spread over a large area due to the long distances traveled by migratory birds, the mixed species flocks in which they congregate, and local concentrations of many species around bird feeders is of great concern to many ornithologists and ecologists, as well as poultry producers. There are indications that the strain, while highly pathogenic to chickens, is not readily transmissible to poultry under natural conditions (Nettles 1996). A new species of mycoplasma (*M. sturni*) was isolated from the conjunctiva of a European starling (*Sturna vulgaris*) with conjunctivitis found in Connecticut during the epornitic (Forsyth et al. 1996). Although it was isolated in pure culture, it was described as growing rapidly, whereas pathogenic mycoplasmas typically grow slowly; therefore, it could have overgrown *M. gallisepticum* if that species had been present. The pathogenicity of *M. sturni* needs to be investigated.

Three new species of mycoplasmas have recently been described from raptors in Europe (Poveda et al. 1994). All were associated with respiratory diseases clinically and histologically consistent with those caused by *Mycoplasma* spp., with lesions including hyperplasia of mucous glands, lymphoid hyperplasia, and perivascular cuffing (Poveda et al. 1990). The species were isolated from buzzards (*Buteo buteo*), saker falcons (*Falco cherrug*), and griffon vultures (*Gyps fulvus*), and have been named, respectively, *M. buteonis*, *M. falconis*, and *M. gypis*. Pathogenicity of these species, their distribution in

wild, free-ranging birds, and their potential impacts on the populations need to be further investigated.

Several species of mycoplasmas, including *M. cloacale* (Bradbury et al. 1987, Goldberg et al. 1995) and *M. anatis* (Ivanics et al. 1988, Poveda et al. 1990, Goldberg et al. 1995), have been isolated from semi-domestic and wild ducks and other avian species throughout the world. Stipkovits et al. (1986) reported isolation of *M. cloacale* from geese with inflammation of the cloaca and phallus, but Goldberg et al. (1995) found no association of *M. cloacale* with disease in wild mallards (*Anas platyrhynchos*), black ducks (*A. rubripes*), or canvasbacks (*A. valisneria*). Stipkovits (1979) reported pathogenicity of *M. anatis* to domestic ducklings and eggs, and neurological signs have been recorded in ducks infected with *M. anatis* (Ivanics et al. 1988). Samuel et al. (1995) infected game-farm mallard eggs with *M. anatis* and found reduced hatchling success, hatchling size and growth rates. Hatchlings infected at 1 d of age did not have slower growth rates. Goldberg et al. (1995) found esophagitis, tracheitis, and vaginitis in female mallards from which *M. anatis* was isolated, and presented evidence for vertical transmission of *M. anatis* in a wild gadwall (*Anas strepera*). Potentially, *M. anatis* infections could reduce recruitment in wild duck populations. Experimental infection of ducklings with *M. gallisepticum* resulted in suppressed growth rates (Stipkovits 1979). Four other unidentified mycoplasmas were isolated from mallards, gadwalls, and black ducks, but could not be associated definitively with disease (Goldberg et al. 1995).

Although *M. gallisepticum* has been isolated from wild turkeys, most cases have occurred in birds with close association to domestic poultry (Davidson et al. 1982, Jessup et al. 1983, Luttrell et al. 1991, Fritz et al. 1992). Even though experimental infection of

pen-reared wild turkeys has resulted in decreased productivity (Rocke et al. 1988), there is little evidence to indicate that infection with *M. gallisepticum* strains commonly occurring in domestic fowl poses a threat to wild turkey populations (Luttrell et al. 1991), or that wild turkey populations are important in the epizootiology of *M. gallisepticum* (Davidson et al. 1988). *Mycoplasma gallopavonis* has been isolated from wild turkeys in Texas (Rocke and Yuill 1987), South Carolina, Georgia (Luttrell et al. 1991), Colorado, New Mexico, and Oklahoma (Fritz et al. 1992). Although lethal to experimentally infected domestic turkey eggs (Rocke and Yuill 1987), the pathogenicity of *M. gallopavonis* to wild turkeys has not been investigated. *Mycoplasma synoviae* and other, untyped, *Mycoplasma* spp. were isolated from turkeys in Arizona (Fritz et al. 1992), but no association with disease was found. As with *M. gallisepticum*, wild turkeys do not appear to be important in the epizootiology of *M. synoviae* or *M. meleagridis* (Davidson et al. 1988).

In 1993 an epizootic of polyarthritis occurred in juvenile farmed crocodiles (*Crocodylus niloticus*) in Zimbabwe. The outbreak was characterized by high morbidity, but low mortality. A mycoplasma was isolated, determined to be a previously unrecognized species, and named *M. crocodyli* (Mohan et. al 1995). In 1995 a die-off of captive adult American alligators (*Alligator mississippiensis*) at a private facility in Florida, with very high mortality, was associated with systemic infection with a different species of mycoplasma, also previously unrecognized (Brown et al. 1996a). The name *M. lacerti* has been proposed. In addition to *M. agassizii*, a second mycoplasma was found in desert tortoises with evidence of URTD, including clinical signs, histologic lesions, and/or positive ELISA tests (Brown et al. 1995). In a small pilot study, that organism was found

to cause disease and seroconversion in gopher tortoises previously unexposed to either mycoplasma (D. R. Brown, et al., unpub data).

Mycoplasmas have been isolated from bighorn sheep (*Ovis canadensis*) with pneumonia associated with *P. haemolytica*, and there is concern that these may have a role in the disease process (D. Hunter, pers. comm.). A mycoplasma isolated from dead and live captive bighorn sheep with pneumonia was typed as *M. arginini* (Woolf et al. 1970). Apparently, no further work has been done regarding the pathogenicity of that strain to bighorn. With continued exposure of bighorn to domestic livestock, *Mycoplasma* spp. could interfere with recovery efforts. An outbreak of pneumonia associated with *M. ovipneumoniae* infection occurred in captive Dall's sheep (*Ovis dalli dalli*) following indirect exposure to domestic sheep (Black et al. 1988). Although Dall's sheep are unlikely to be exposed to domestic livestock in their native habitat, it is clear that captive herds or those in transit must be protected from exposure to pathogens.

Behymer et al. (1989) detected antibodies to *Mycoplasma* spp. in mule deer (*Odocoileus hemionus*) in California, but there was no association with disease, and isolations were not attempted. One-humped camels (*Camelus dromedarius*) and African buffalo (*Syncerus caffer*) on a game farm in Kenya had antibodies against *Mycoplasma* strain F38, and camels were seropositive for *M. mycoides mycoides*, but no isolations were made and no disease was seen (Paling et al. 1988). Kirchoff et al. (1996) found, in a survey of captive arthritic elephants (*Elephas maximus* and *Loxodonta africana*), that about 60% of the females harbored a new mycoplasma in the urogenital tract, although none was recovered from males. It is not known if the mycoplasma, named *M. elephantis*, causes arthritis.

Chronic Manifestations of Mycoplasmal Infections

Many mycoplasmal diseases are characterized by an overaggressive or inappropriate immune response by the host, eventually leading to autoimmune damage to the affected sites, whether respiratory or urogenital tract, joints, heart, skin, or other organ systems (Krause and Taylor-Robinson 1992, Simecka et al. 1992, Cole 1996). Infected hosts may be more susceptible to secondary infections with other bacteria or viruses. In wildlife species, such complications may reduce the fitness of the animals by altering behavior, leading to decreased foraging efficiency, increased susceptibility to predators, or diminished mate seeking behavior. Energy that normally would be allocated to reproduction may be needed to repair or compensate for damage to multiple organ systems. Therefore, even if mycoplasmal infections do not cause mortality directly, they can affect individual and population viability.

Project Overview and Specific Objectives

Due to the listing by the Florida Game and Fresh Water Fish Commission of the gopher tortoise as a species of special concern, and the subsequent permitting of over 450 relocations involving more than 8000 tortoises, particular attention has been focused on the dynamics and persistence of both natural and relocated populations. Understanding the effects of URTD on individuals and populations is essential for proper management of remaining populations; therefore, a study was begun in 1993 on the etiology, pathology, and diagnosis of URTD in gopher tortoises. The original objectives were 1) to describe the pathology of natural infections and identify possible etiologic agents, 2) to perform

experimental infections to determine conclusively the etiologic agent and dose-related effects, 3) to refine the PCR test and delineate further the taxonomy of the tortoise mycoplasmas, 4) to characterize the immune responses of gopher tortoises to URTD pathogens, and 5) to compare the efficacy of culture, PCR and serology in detecting new infections.

As the original experiments were conducted and concern over the continued well-being of tortoise populations throughout the range increased, additional questions were raised. Those were concerned primarily with management and conservation of gopher tortoises, particularly with respect to relocation, repatriation, and restocking efforts. Several questions were raised repeatedly in meetings with state and federal agency personnel, particularly regarding possible immunity to the organism and factors related to transmission. In order to address those questions additional objectives were established, as delineated in specific objectives 2-7 below, and discussed in Chapters 3 through 8. Chapter 2 will detail the methods common to fulfilling the objectives.

This dissertation addresses the following specific objectives:

1) To describe the clinical presentation and lesions of natural infections by *M. agassizii* in gopher tortoises and compare the lesions to those found in desert tortoises (Chapter 3).
2) To test the hypothesis that tortoises that have produced antibodies against *M. agassizii* are protected against reinfection with the organism and subsequent development of URTD (Chapter 4).
3) To assess the probability of horizontal transmission of *M. agassizii* between adult tortoises and to test the hypothesis that transmission is more likely to occur from an

infected host that is clinically ill and PCR or culture positive than from a seropositive host that is not clinically ill or is clinically ill but culture or PCR negative (Chapter 5).

4) To determine if *M. agassizii* is transmitted vertically (Chapter 6).
5) To assess the relationship of antibodies in eggs and hatchling serum to those in maternal serum (Chapter 6).
6) To collect preliminary data to test the hypothesis that *M. agassizii* can be transmitted environmentally (e.g., in burrows) (Chapter 7).
7) To discuss the implications of the findings of the above research and additional concurrent research on conservation and management of gopher and other tortoises (Chapter 8).

CHAPTER 2
METHODS

Tortoises, Intake Procedures, Clinical Assessments and Sampling Methods

For the natural infection studies, tortoises were obtained from various locations in the state of Florida under Florida Game and Fresh Water Fish Commission permits number WX93227 issued to Elliott R. Jacobson and number WX94037 issued to Mary B. Brown. Tortoises were processed the day of arrival at the University of Florida (UF), Gainesville. For the natural and experimental infection studies, gopher tortoises were transferred from a development site in central Florida to UF in April, July, and August 1994 and April 1995, and processed the day following arrival. Tortoises were examined for clinical signs of URTD: nasal and ocular discharge, palpebral edema, and conjunctivitis. The signs were graded individually on a scale of 0-3, none to severe. Total clinical sign score was calculated as the nasal discharge score plus the mean of three ocular sign scores (ocular discharge, palpebral edema, and conjunctivitis). Tortoises were weighed to the nearest 10 g, and ketamine hydrochloride (Ketaset®, Fort Dodge Laboratories, Inc., Fort Dodge, Iowa) was administered at 20 mg/kg. Straight line carapace length, thickness, and width were measured to the nearest cm with forestry calipers.

A 2-3 ml blood sample was drawn from the jugular or brachial vein and placed in a lithium heparin Vacutainer tube (Becton Dickinson and Company, Rutherford, New Jersey). Blood was centrifuged and an aliquot of plasma was removed for antibody screening by an enzyme-linked immunosorbent assay (ELISA) (see below).

After cleansing the area around the nares with alcohol dampened gauze, nasal flush samples were collected by flushing with approximately 0.5 ml sterile SP4 broth using a 1 ml syringe without needle. Calcium alginate-tipped swabs were inserted gently into the nares, and a sample was obtained and streaked onto SP4 agar plates.

Culture Procedures

A 100 µl aliquot of the flush sample was used for polymerase chain reaction (PCR) analysis; the remaining sample was serially diluted ten-fold to 10^{-2} and incubated at 30°C for a maximum of three weeks, or until determined to be positive or contaminated. Twenty µl of each dilution were placed on SP4 agar and incubated at 30°C and 5% CO, as were the streak plates. Plates were examined regularly for a maximum of six weeks to detect the growth of mycoplasma. In the second year of the study, broth cultures were incubated for 24-48 hr before removing the aliquot for PCR, as that modification increased the sensitivity of the PCR (G. S. McLaughlin and D. R. Brown, unpub. data).

PCR Procedure

Nasal aspirate samples were analyzed for the presence of *Mycoplasma agassizii* DNA based upon PCR amplification of the 16S rRNA gene (Brown et al. 1995). Nasal flush and culture samples were centrifuged for 60 min at 14,000 rpm at 4°C, and the

supernatant aspirated. Three to four microliters of 20 µg/ml proteinase K (Sigma, St. Louis, MO) in 20 µl lysis buffer (100 mM tris pH 7.5, 6.5 mM DTT, 0.05% Tween 20) were added to the pellets, which were resuspended, and the samples were incubated at 37°C for 8-16 hours. After denaturing the proteinase K at 97°C for 15 min, 5 µl of each sample were removed and added to 45 µl of reaction solution containing two primers for the 16S rRNA gene at 1 µM each, deoxynucleoside triphosphates at 200 µM, 2.0 mM MgCl, and 2.5 units of *Taq* polymerase (Promega, Madison, WI). The primers were complementary to sequences found in the V3 variable region of the 16s rRNA gene (sense strand nucleotides (nt) 471 to 490, 5′-CCTATATTATGACGGTACTG-3′, Brown et al. 1995) and a *Mycoplasma* genus-specific region [anti-sense strand nt 1055 to 1031, 5′-TGCACCATCTGTCACTCTGTTAACCTC-3′, Van Kuppeveld 1992]. Samples were subjected to 50 cycles of template denaturation for 45 sec at 94°C, primer annealing for 1 min at 55°C, and polymerization for 45 sec at 72°C, followed by 10 min at 72°C. Positive samples yielded 576 base pair (bp) products that were visualized by combining 15 µl of product with 2 µl bromphenol blue in 50% glycerol solution and electrophoresing on ethidium bromide-stained 1.5% agarose gels in tris-borate-EDTA buffer. Positive control samples using 250 ng of purified *M. agassizii* DNA as the template and negative control samples, with water in place of a template, were included with each amplification run. A molecular weight marker, Hind III digest of $\phi\chi$ phage DNA, was included on each gel.

In order to confirm that the isolates obtained from naturally and experimentally infected tortoises were *M. agassizii*, an additional procedure, restriction fragment length polymorphism (RFLP) analysis, was conducted on at least one isolate from each tortoise. Twenty microliter samples of products from the above amplification procedure were

incubated with 10-20 units of the endonuclease AgeI (New England Biolabs, Inc., Beverly, MA), which cuts the *M. agassizii* amplification product at nt 613, and 5 µl of reaction buffer, at 25°C for one hour, and the products electrophoresed as above. The procedure resulted in products of 434 and 142 bp from *M. agassizii*-positive samples, and no change in non-*M. agassizii*-samples.

ELISA Procedure

An aliquot of plasma from each sample was used for determinations of levels of antibodies specific for *M. agassizii* (Schumacher et al. 1993). Ninety-six-well microtiter plates (Maxisorp F96, Nunc, Kamstrup, Denmark) were coated with 50 µl of a whole-cell lysate of *M. agassizii* strain 723 at 10µg/ml in phosphate buffered saline with 0.02% azide (PBS-AZ). Plates were incubated overnight at 4°C, washed four times with PBS-AZ plus 0.05% Tween 20 (PBST) in an automatic plate washer (EL403, Bio-Tek Instruments, Inc., Winooski, VT), and blocked overnight at 4°C with 250 µl/well PBST containing 5% non-fat dry milk (PBS-TM). Following washing, 50 µl of plasma diluted appropriately for the specific study with PBS-TM were added to individual wells in duplicate or triplicate, and the plates were incubated at room temperature for 60 min. The plates were washed, 50 µl/well of a biotinylated monoclonal antibody (MAb HL673) against the light chain of desert tortoise immunoglobulins IgY and IgM at 1 µg/ml in PBS-TM was added, and plates were incubated for 60 min. Following washing, a conjugate of alkaline phosphatase and streptavidin (AP-S; Zymed Laboratories, Inc., San Francisco, CA) at 1:2000 in PBS-AZ was added at 50 µl/well, plates were incubated for 60 min, and washed. Substrate, *p*-nitrophenyl phosphate disodium (*p*NPP; Sigma), was prepared at 1 mg/ml in 0.01 M

sodium bicarbonate, pH 9.6, with 2 mM $MgCl_2$, and added to wells at 100 µl/well. Plates were incubated for 60 min in the dark, then read at 405 nm on a microplate reader (EAR 400 AT, SLT Labinstruments, Salzburg, Austria). The mean of two or three wells coated with antigen and incubated with conjugate and substrate only was used as the blank. A positive control, plasma from a naturally infected gopher tortoise from Sanibel Island, and a negative control, plasma from an uninfected tortoise from Orange County, were included on each plate.

Results from the ELISA were optical density (OD) readings from the microplate reader. The OD readings reflected the intensity of the yellow color developed when all components of the reaction (specific tortoise antibodies against *M. agassizii*, biotinylated MAb HL673, AP-S, and *p*NPP) were present. The readings are on a continuous scale, but were interpreted categorically by calculating the ratio of the sample readings to the negative control reading. Ratio values less than or equal to 2.0 were considered negative, those greater than 2.0 and less than or equal to 3.0 were classified as suspect, and those greater than 3.0 were classified as positive.

Study Group Assignment

Tortoises exhibiting one or more signs of disease, or with a positive culture or PCR result, were designated as diseased. Animals were designated clinically healthy if found free of signs of URTD, and with negative culture and PCR results. Tortoises were assigned to study groups based on sex, weight, clinical assessments, culture, PCR and ELISA results, and placed in the appropriate pens.

Husbandry Procedures

Tortoises were housed singly or in pairs in outdoor pens at the UF Animal Resource Farm (ARF). There were four groups of 10 pens in a larger, chain link-fenced enclosure (Fig. 2-1). Pens were approximately 21 m^2, constructed of a wooden frame with sheet metal extending vertically approximately 0.7 m above and below ground, partially covered by shade cloth, and provided with an artificial burrow, a water dish, and a cement feeding stone. Pens were observed daily by ARF staff, and watered daily in the summer and as needed in the winter. The tortoises were fed a salad of mixed vegetables three times per wk, and fruit was provided on an occasional basis. In addition, I observed the pens and tortoises from three to seven days each week, with some days including multiple observations. Because of individual behavior patterns, not every tortoise was observed at each time point. The amount of food eaten was recorded for each pen the day following feeding, and the stones were cleaned. Remaining food was collected and bagged separately for each section, using brushes and dust pans assigned to that section. Stones were then sprayed with a dilute (1:30) bleach solution, allowed to soak for a few minutes, and hosed off. Water dishes were rinsed and filled daily, and bleached and scrubbed as necessary. Husbandry personnel wore gloves for all procedures requiring handling of food, feeding stones, or water dishes.

Entry into pens and handling of tortoises were restricted to research personnel. Any person handling a tortoise wore clean gloves, which were changed as necessary and before handling a different tortoise. Used gloves were placed in plastic garbage bags and disposed of properly. Before entering the first pen on a given day, personnel sprayed their footwear with a dilute bleach solution. Footwear was sprayed with bleach before leaving

a pen. Animals were captured by hand or using wire cage-type traps (Tomahawk Live Trap Company, Tomahawk, WI) that were covered with brown paper to protect the animals from the weather. Traps were cleaned, sprayed with bleach solution, and allowed to air dry following each use. Paper was discarded, and fresh paper was used for the next trapping effort. Each tortoise was placed in a plastic, lidded container (LEWISystems, Menasha Corporation, Watertown, WI) for transport and holding. Containers were bleached, scrubbed, and washed in an automatic cage washer before reuse.

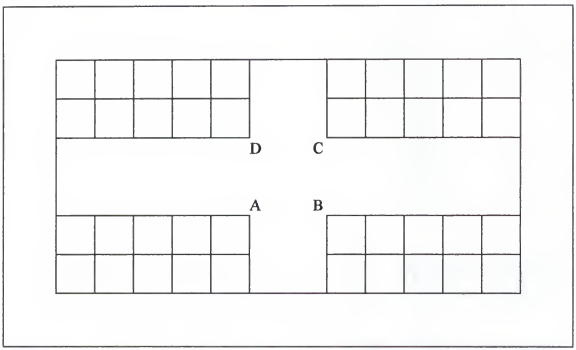


Figure 2-1. Layout of tortoise pens at the University of Florida Animal Resource Farm. The outer fence was chain link, and the inner fence and pen dividers were corrugated sheet metal on a wooden frame.

Necropsy Procedures

All diseased and selected healthy tortoises were euthanatized with a combination of drugs. Ketamine was administered intramuscularly at 60 - 80 mg/kg followed by a

concentrated barbiturate solution (Socumb, The Butler Company, Columbus, Ohio, USA) intraperitoneally at 1 ml/kg. Once the tortoises showed complete muscle relaxation and were unresponsive to painful stimulation, they were exsanguinated via a 23 gauge butterfly catheter inserted into the carotid artery and then decapitated. Flush and swab samples were collected as previously described, then the head was bisected longitudinally with an electric saw. Following bisection, the cartilage over each nasal cavity was reflected aseptically, and flushes and swabs of both left and right nasal cavities were collected.

For those tortoises selected for complete necropsy, the plastron was removed from the carapace, and viscera within the coelomic cavity were exposed. A gross necropsy was conducted and the following tissues were collected, fixed in neutral buffered 10% formalin, sectioned at 5-6 μm, and stained with hematoxylin and eosin: glottis, cranial trachea, tracheal bifurcation, left lung, right lung, thyroid, heart, brain, thymus, esophagus, stomach, small intestine, pancreas, large intestine, cloaca, spleen, liver, left and right kidney, bladder, right and left gonads, chin gland, buccal salivary gland, chin gland, and tongue. Tissues were examined by light microscopy and abnormalities or changes were recorded.

Histopathology Procedures

For histopathologic studies, heads were fixed in 10% neutral buffered formalin (NBF), decalcified, embedded in paraffin, sectioned longitudinally at 5-6 μm, and stained with hematoxylin and eosin. Sections were examined by light microscopy and classified on a scale of 0 to 5, with 0 being normal and 5 exhibiting severe inflammation and / or changes. Changes in the epithelium and submucosa were recorded separately.

The following criteria were utilized for grading lesions:

Normal (score = 0): Occasional small subepithelial lymphoid aggregates; rare heterophils in the lamina propria. No changes in mucosal or glandular epithelium; no edema.

Mild (1): Multifocal small subepithelial lymphoid aggregates; multifocally, small numbers of heterophils, lymphocytes, and plasma cells in the lamina propria; mild edema in lamina propria; minimal changes in mucosal epithelium.

Moderate (2-3): Multifocal to focally extensive lymphoid aggregates; diffusely, moderate numbers of heterophils, lymphocytes, and plasma cells in the lamina propria, occasionally infiltrating the overlying mucosal epithelium; moderate edema in the lamina propria; proliferation and disorganization of the basal epithelium.

Severe (4-5): Focally extensive to diffuse band of lymphocytes and plasma cells subjacent to and obscuring the overlying mucosal epithelium; large numbers of heterophils in lamina propria and infiltrating overlying mucosal epithelium; marked edema of the lamina propria; degeneration, necrosis, and loss of the mucosal epithelium with occasional erosion; proliferation of the basal cells of the epithelium with metaplasia of the mucous and olfactory epithelium to a basaloid epithelium; occasional squamous metaplasia.

Statistical Analyses

Statistical analyses were performed using SAS (SAS Institute, 1988) or SigmaStat for Windows, Version 1.0 (Fox et al. 1994). Because the analyses varied for each experiment, the specific methods will be addressed in the appropriate chapters.

CHAPTER 3
NATURALLY OCCURRING UPPER RESPIRATORY TRACT DISEASE

Methods

Tortoises

Twenty-three gopher tortoises from the following locations in Florida (Figure 3-1) were transported to UF from August 1993 to September 1995: Alachua County ($n = 2$), Sanibel Island, Lee County ($n = 3$), Volusia County ($n = 1$), St. Lucie County ($n = 1$), Indian River County ($n = 1$), Orange and/or Osceola Counties ($n = 15$). Collection of tortoises, except those from Orange and Osceola Counties, was opportunistic, and a result of submissions to the UF Wildlife Clinic, or other veterinary clinics. Some tortoises had exhibited signs of URTD, while others had been hit by automobiles. Tortoises from Orange and Osceola Counties were selected on the basis of clinical evaluations, ELISA, culture, and / or PCR results. Six clinically healthy animals were included in the latter group. Tortoises were evaluated for clinical signs of URTD (i.e., nasal and ocular discharge, palpebral edema, and conjunctivitis) and those exhibiting one or more signs of disease, with a past history of clinical signs, with positive culture or PCR results, or with a positive ELISA result, were designated as diseased. Tortoises were designated healthy if they were free of any history of or current signs of URTD and were culture, PCR, and ELISA negative.

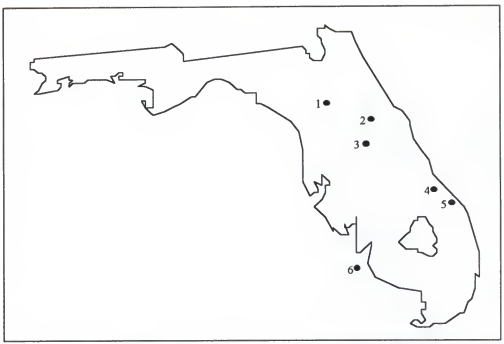


Figure 3-1. Locations in Florida from which gopher tortoises were obtained. 1 - Alachua County, 2 - Volusia County, 3 - Orange/Osceola Counties, 4 - Indian River County, 5 - St. Lucie County, 6 - Sanibel Island, Lee County.

Necropsy and Histology Procedures

Necropsy procedure and light microscopic evaluation of tissues were performed as detailed in Chapter 2. Gross necropsies were conducted on 21 tortoises. Multiple tissues were collected from 15 tortoises, heads and livers from six , and only heads from two.

Microbial Isolation

Flush and swab samples for *Mycoplasma* isolation were collected from the nasal passages and cavities of each tortoise and processed as described in Chapter 2. Swab specimens of the dorsal nasal cavities of 16 tortoises were collected for aerobic bacteria isolation and submitted to the Clinical Pathology Laboratory (CPL) of the College of Veterinary Medicine (CVM) at UF. Samples were cultured on Columbia blood agar and MacConkey's agar, and incubated at 37°C. Bacteria were identified utilizing the

identification systems API 20E for enteric organisms and API NFT for non-enterics (BioMerieux Vitek, Hazelwood, MO, USA). Isolates of organisms consistent with *Pasteurella* were identified to species according to biochemical profiles listed for *P. testudinis* (Snipes and Biberstein 1982).

Electron Microscopy

Selected specimens were submitted to H. P. Adams of New Mexico State University, Las Cruces, for scanning and transmission electron microscopic preparation and evaluation.

The left half of the bisected head of one healthy tortoise was prepared for ultrastructural evaluations. The nasal cavity was instilled with 2.5% glutaraldehyde in 0.1 M phosphate buffer, then dissected out in its entirety, and selected areas were sampled. The tissues were dehydrated in an ascending series of ethanols and transferred to hexamethyldilisilazane for the final drying. The samples were sputter coated with gold and viewed by scanning electron microscopy (SEM).

Samples from 11 diseased tortoises were collected for transmission electron microscopy (TEM). The nasal cavity tissue was removed from the underlying cartilaginous tissues and separated into anterior dorsal, anterior ventral, posterior dorsal and posterior ventral quadrants. Each quadrant was cut into 1 mm cubes and placed in 2.5% glutaraldehyde, and post-fixed in osmium tetroxide. Specimens were prepared for TEM by embedding in epon-araldite and sectioning with an ultramicrotome. Thick sections were stained with toluidine blue and examined by light microscopy. Ultrathin sections were placed on copper grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi H7000 transmission electron microscope.

Results

Based on clinical evaluations and diagnostic tests, eight tortoises were classified as healthy and 15 as affected by URTD. Infected tortoises were from Orange / Osceola Counties (n = 10), Lee County (n = 3), Indian River County (n = 1) and St. Lucie County (n = 1). Histological findings for the two groups will be discussed separately.

Normal Anatomy and Histology

The external nares opened into ventro-lateral depression, and were continuous with large dorsal nasal cavities (Figure 3-2). Right and left dorsal nasal cavities were separated by a cartilaginous septum. Each nasal cavity was bisected by a ridge, forming anterior and posterior compartments. Ventrally, the nasal passageways were continuous with the choanae (internal nares), which opened into the palatine region of the dorsal oral cavity.

The integument continued through the external nares into a short vestibule, which was initially lined by keratinized stratified squamous epithelium. That epithelium abruptly changed to mucous glandular epithelium, which lined the nasal passageway throughout its length. Interspersed among the mucous epithelial cells were ciliated epithelial cells. The ventro-lateral depression was lined primarily by mucous and ciliated epithelial cells (Figure 3-3a). Both anterior and posterior dorsal nasal chambers were lined by a multilayered olfactory epithelium with occasional mucous cells (Figure 3-3b). Numerous serous and mucous glands, vessels, nerve bundles, and clusters of melanophores were present in the connective tissue surrounding the nasal cavities. Small focal aggregates of lymphoid cells were seen in the submucosa.

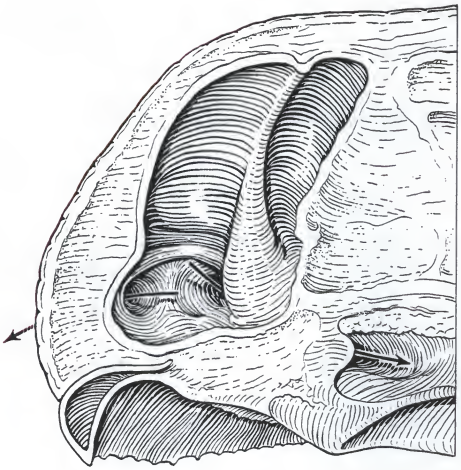
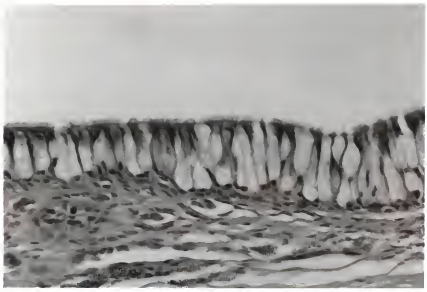


Figure 3-2. Diagrammatic representation of the interior of a gopher tortoise head sectioned longitudinally, illustrating the relationship of the nasal cavity to the external and internal nares. Approximately 3x life size. Drawing by L. Mallory.

a



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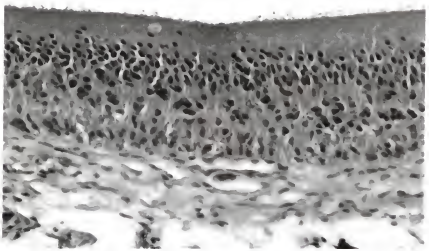

Figure 3-3. Photomicrographs of normal gopher tortoise upper respiratory tract tissues. Hematoxylin and eosin staining, 320x. a) Photomicrograph of the ventro-lateral depression demonstrating the ciliated mucous epithelium; b) Photomicrograph of the dorsal nasal cavity demonstrating the multilayered olfactory epithelium. Photographs by E. R. Jacobson.

The bilateral thymus glands were difficult to find in healthy tortoises and were located cranial to the base of the heart, at the branching of the subclavian and carotid arteries. Grossly, the thymus was multilobulated. Histologically, there was a typically dark staining cortex and a lighter staining medulla. The cortex contained densely packed thymocytes. In the medulla there were significantly fewer cells including thymocytes, as well as thymic epithelial cells, myoid cells, and heterophils.

The spleen was located on the right side, between the proximal duodenum and transverse colon and was associated closely with pancreatic tissue. Histologically, spleens were composed of distinct areas of white and red pulp. White pulp consisted of collections of lymphoid tissue surrounding blood vessels. Red pulp, located between the perivascular collections of the white pulp, included red blood cells within sinusoids and small numbers of lymphocytes.

The thyroid was located at the base of the heart. In one of the healthy tortoises, the thyroid was enlarged, with multifocal areas of follicular epithelial cell hyperplasia. In all tortoises, follicles varied in size, with many having numerous red blood cells in the colloid and either intraepithelial or supra-epithelial vacuoles.

Multiple foreign bodies were seen in the submucosa of the glottis of two tortoises, in the tongue of one tortoise, and in the buccal salivary gland of one tortoise. The foreign bodies were consistent with plant material. Lymphoid aggregates were scattered throughout the esophagus, small intestine, large intestine and cloaca, and also were present in the connective tissue surrounding the mental (chin) glands.

Of the healthy tortoises that were examined fully at necropsy, three were males and three were females. In one male, multifocal areas of mineralization of seminiferous tubules

were seen. Golden brown granules were scattered throughout the kidney within renal tubular epithelial cells of all tortoises. Mononuclear cells containing similar appearing granules were within the renal interstitium and the interstitium of the testes.

Pathologic Findings

Gross examination of heads of diseased tortoises revealed minimal to large amounts of exudate within the nasal cavity and nasal passageways. Histologically, of the 15 heads, one had no changes, two had mild changes, seven had moderate changes, and five had severe inflammatory changes. In the tortoises with minimal changes, mild mucosal hyperplasia and slightly increased lymphoid aggregates were seen in the nasal passage and ventro-lateral depression. In those tortoises with moderate changes, the olfactory epithelia were usually normal, with only focal or mild changes in the submucosa. Changes generally were confined to the nasal passage and the ventral aspects of the nasal cavities, and consisted of mucosal epithelial and lymphoid hyperplasia, with infiltration of mononuclear cells and heterophils. In some tortoises with moderate inflammation, basal cell proliferation and loss of cilia could be detected. In the tortoises with severe inflammatory changes, there were lymphoid aggregates around submucosal glands, with glandular epithelial hyperplasia. The normal mucosal architecture was replaced by infiltrates of mononuclear cells and heterophils. The olfactory mucosa was replaced with proliferating mucous epithelial cells (Figure 3-4). Proliferating basal cells projected into the underlying lamina propria of some tortoises. Exudate, consisting of sloughed epithelial cells and inflammatory cells, was found in the nasal cavity lumen.

Infected tortoises had larger lymphoid aggregates in the submucosa of the glottis; several tortoises had focal areas of epithelial cell proliferation. Basal cell proliferation and

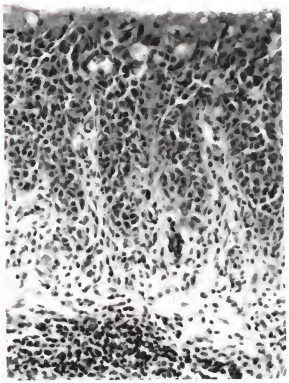

Figure 3-4. Photomicrograph of the nasal cavity tissues of a gopher tortoise with upper respiratory tract disease. The changes were classified as severe, with aggregates of lymphoid cells in the submucosa, proliferation of the basal cells, and dysplasia of the mucosal epithelium. Hematoxylin and eosin staining, 320x. Photograph by E. R. Jacobson.

submucosal lymphoid hyperplasia were seen in the glottis of one tortoise. The hyperplasia extended into the cranial tracheal epithelium. In that tortoise, there also were multifocal areas of epithelial cell hyperplasia in the lung. Five other diseased tortoises had focal to multifocal lymphoid aggregates in the lung interstitia.

The gastrointestinal tracts tended to have increased numbers and larger lymphoid aggregates in the submucosa compared to those of clinically healthy tortoises. In one tortoise there was mucous cell hyperplasia of the colon and in another there was a severe colitis with basal epithelial cell hyperplasia and submucosal lymphoid hyperplasia, with infiltrates of large numbers of heterophils. Four other tortoises had increased lymphoid aggregates in the esophagus, stomach and/or small intestine. Three additional tortoises had increased lymphoid aggregates in the submucosa of the cloaca.

The kidneys of all diseased tortoises contained golden brown pigment granules within renal epithelial cells. Hepatocytes of most tortoises contained similar granules, and pathologic changes were seen in the livers of nine tortoises. In seven, there were increased numbers and size of melanomacrophages in the liver and increased amounts of golden brown granules. One tortoise had cuffing of the central vein by lymphocytes and heterophils; another had aggregates of lymphocytes and melanomacrophages.

Electron micrographs of tissues of one tortoise from Sanibel Island and the tortoise from Indian River County demonstrated organisms consistent with *Mycoplasma* on the surface of the nasal mucosa (Figure 3-5). Associated epithelial cells had vacuolated cytoplasm and inflammatory cell infiltrates were present in the mucosa. Increased numbers of mucous epithelial cells were seen, consistent with light microscopic findings.

ELISA and PCR Results

ELISA and PCR results for both healthy and diseased tortoises are presented in Table 3-1. All healthy gopher tortoises were seronegative for antibody specific against *M. agassizii*. Twelve diseased tortoises were seropositive, two were suspect, and one was seronegative. All healthy tortoises were PCR negative while 11 diseased tortoises were PCR-positive for *Mycoplasma* in nasal aspirates. Four clinically healthy tortoises that had negative culture and PCR results from nasal passage flush and swab samples had positive cultures and / or PCR results for samples from the nasal cavities.

Microbial Isolation Results

The results of *Mycoplasma* and aerobic microbial cultures of the upper respiratory tract (URT) are presented in Tables 3-1 and 3-2. *Mycoplasma* was not cultured from the URT of any healthy tortoise. *Mycoplasma* was cultured from the URT of 11 tortoises with URTD. The aerobic microbial isolates of healthy tortoises consisted primarily of members of the genera *Staphylococcus, Streptococcus,* and *Corynebacterium*; a few Gram-negative rods were isolated. A greater number of Gram-negative species were isolated from the nasal cavities of tortoises with URTD, and those isolates made up a greater proportion of the isolates. *Pasteurella testudinis* was isolated from five diseased tortoises; in two it represented the major aerobic isolate.

Discussion

Gopher tortoises with clinical signs of URTD and evidence of exposure to *Mycoplasma agassizii* were obtained from multiple sites in Florida. The light microscopic

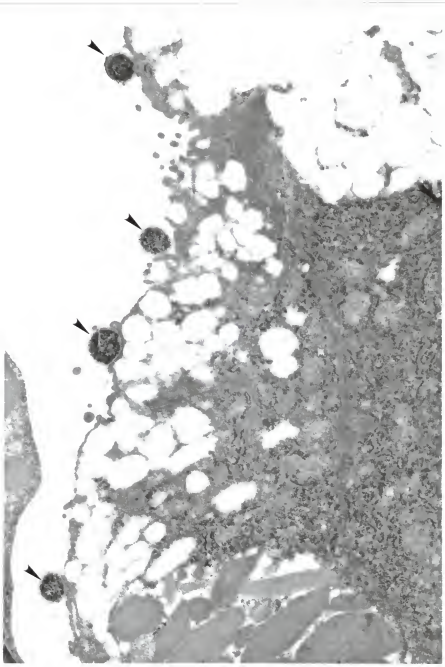

Figure 3-5. Transmission electron photomicrograph of the nasal cavity mucosa of a gopher tortoise with upper respiratory tract disease. Organisms consistent with *Mycoplasma* (arrow) can be seen in close association with host cell membranes. Magnification 18,000x. Photograph by H. P. Adams.

Table 3-1. Summary of ELISA, PCR, culture, and nasal histopathology results from necropsied gopher tortoises from various locations in Florida, for determining infection with *Mycoplasma agassizii*.

Group	Clinical Signs	ELISA	PCR	*Mycoplasma* Culture	Histopathology
Normal ($n = 8$)	0%	0%	0%	0%	0%
Natural Infection ($n = 15$)	60%	85%	92%	80%	92%

Table 3-2. Summary of aerobic culture results from nasal cavity swabs of gopher tortoises from Florida.

Group	Growth	Species	Percent of isolates
Normal ($n = 5$)	very scant to moderate	*Staphylococcus* spp. Gram-negative rods *a*-hemolytic *Streptococcus* sp. non-hemolytic *Streptococcus* spp. *Corynebacterium* spp.	45 25 10 10 10
Natural Infection ($n = 11$)	very scant to heavy	*Corynebacterium* spp. *Pasteurella testudinis* *Staphylococcus* spp. Gram-negative rods *Micrococcus* sp. *Flavobacterium, Pseudomonas, Lactobacillus,* and others	33 18 16 15 11 7

changes in the upper respiratory tract of the gopher tortoises were similar to inflammatory and dysplastic changes reported for desert tortoises with URTD (Jacobson et al. 1991, Brown et al. 1994, Jacobson et al. 1995). However, inflammationa and epithelial proliferation around the glottis, tracheitis, and proliferative pneumonia have not been seen in desert tortoises. Additionally, two diseased tortoises had proliferation of the colonic mucosal epithelium. Those changes have not been seen in desert tortoises with URTD.

By electron microscopy, organisms consistent with *Mycoplasma* were demonstrated on the nasal mucosal surfaces of two tortoises. Other than bacteria, no infectious agents were demonstrated in or on nasal cavity mucosa by electron microscopy. Eleven diseased gopher tortoises were PCR positive and *M. agassizii* was cultured from the upper respiratory tract of 11 diseased tortoises examined. These results support the hypothesis that *M. agassizii* is a cause of URTD in gopher tortoises in Florida.

The greater number of species and increased proportion of Gram-negative bacteria isolated from diseased tortoises could indicate that conditions in the upper respiratory tract of diseased tortoises are more favorable for the growth of those species, or that tortoises infected with *M. agassizii* are more susceptibile to opportunistic pathogens. The positive culture and / or PCR results from nasal cavity samples (obtained at necropsy) of four clinically healthy tortoises with negative nasal passage flushes and swabs support the hypothesis that tortoises can harbor the organism without showing clinical signs or shedding bacteria. Such animals may recrudesce under stressful conditions, begin shedding bacteria, and become infective to other tortoises.

Nine gopher tortoises exhibited pathologic changes in the livers, although the significance of these changes is not understood currently. The mycoplasma may release

toxic compounds or induce the production of compounds by the tortoises that cause damage to the liver. Desert tortoises with URTD also show changes in liver tissue (B. L. Homer, pers. comm.), and some exhibit altered nitrogen metabolism (B. Henen, pers. comm.). The altered nitrogen metabolism may be due to behavioral changes leading to decreased foraging rates or efficiency, or it may be due to direct effects of the *Mycoplasma* infection, but the mechanism is not yet understood. Although only one of nine experimentally infected captive gopher tortoises significantly decreased its intake of vegetables (G. S. McLaughlin, unpub. data), and that decrease was temporary, wild tortoises may alter their behavior patterns to a greater degree. Alternatively, secondary infections by other bacteria may be the proximate cause of liver damage. However, no evidence of primary or secondary bacterial infection (i.e., necrosis) was seen histologically.

By the ELISA, none of eight clinically healthy tortoises and 11 of 15 diseased tortoises had antibodies against *M. agassizii*, indicating previous exposure. Tortoises with negative ELISA results may have been in the early stages of the disease, when increased antibody levels had not occurred or were not detectable. The four diseased, seronegative, tortoises may have been infected with another agent. Westhouse et al. (1996) implicated an iridovirus as the cause of pneumonia, tracheitis, pharyngitis, and esophagitis in a gopher tortoise from Sanibel Island. The virus was readily detectable on both light and electron microscopy. It is not known if attempts were made to culture mycoplasma, or if an ELISA was run on a plasma sample. No indications of viral infections were seen in the tortoises examined for the current study.

Investigators recently have found seropositive gopher tortoises in Georgia (B. Raphael, pers. comm.), seropositive, clinically ill, and/or PCR positive tortoises at a site in

northeast Florida, and seropositive tortoises at a site in Mississippi (D. M. Epperson, pers. comm.). Although many Florida and Georgia tortoise populations are fairly large, those in Mississippi are more restricted and are on the federal endangered species list. Outbreaks of URTD in populations with limited recruitment and no nearby source populations could contribute to severe declines in numbers and, possibly, local extinctions. Further studies, on a range-wide basis, need to be conducted to determine the distribution and potential impacts of URTD on gopher tortoise populations.

In addition, further research is necessary regarding the pathogenesis of URTD. While findings of liver, kidney, and intestinal tract lesions are interesting, they do not elucidate the mechanisms by which the changes are caused. Changes have been found in hormonal profiles of some infected desert tortoises (Rostal et al. 1996), which could lead to altered foraging and reproductive behavior, as well as decreased reproductive potential. If foraging behavior is affected, food and water intake might be reduced, which could affect liver and kidney functions. Both direct and indirect pathogenic mechanisms need to be studied in order to better predict the effects of URTD on tortoises.

CHAPTER 4
EFFECTS OF REPEATED EXPOSURE ON SEROPOSITIVE ADULTS

Introduction

In order to properly evaluate the results of the diagnostic tests and incorporate those findings into management and conservation plans, epidemiological questions, including the response of seropositive, asymptomatic tortoises to subsequent exposure to the agent, must be addressed. Although vaccines have been developed for some mycoplasmal diseases (Ellison et al. 1992, Lai et al. 1996, Markham et al. 1996), they are usually not fully protective (e.g., Djordjevic et al. 1996, Kleven et al. 1996, Mohan et al. 1996, Washburn and Weaver 1996), and many natural infections by mycoplasmas do not engender a protective host immune response. The immune response is actually essential to the development of lesions, and infected animals are susceptible to repeated infection (Simecka et al. 1992). Due to the immunopathology, the disease may be more severe on subsequent exposure than on initial infection. I designed an experiment to test the hypothesis that tortoises that have produced antibodies against *M. agassizii* are protected against reinfection with the organism and subsequent development of URTD.

Methods

Acquisition of tortoises, intake and husbandry procedures, clinical assessments, sampling methods, ELISA and PCR procedures, and necropsy procedure were as detailed in Chapter 2.

Statistical Analyses

The onset and severity of clinical signs of URTD, and ELISA, PCR, and culture results for the challenge tortoises were compared to those for control and naive tortoises using the SAS system (SAS Institute, 1988). Data from an additional naive animal infection experiment were included after it was determined that the data did not differ significantly from those collected from naive animals in this study. This ensured large enough sample sizes for meaningful comparisons at more time points. Differences in the severity of clinical signs, histologic lesions, and ELISA data among the three treatment groups were compared by an analyses of variance-type logistic regression using maximum likelihood estimators to compensate statistically for the different number of tortoises in each group at each sampling date. Percentages of tortoises showing clinical signs at different time points were compared by Fisher's Exact test, with a P value of 0.05 accepted as significant.

Experimental Design

Four groups of tortoises were established. Three groups [control ($n = 6$), naive ($n = 11$), and sentinel ($n = 2$)] had no history of exposure to *M. agassizii*, while the fourth, or challenge group ($n = 8$), had previous history of exposure as indicated by a positive result

on the ELISA. Initially, no tortoise in any group had clinical signs of URTD, or positive culture or PCR results. One tortoise originally slated for inclusion in the challenge group (i.e., seropositive, but clinically, culture, and PCR negative upon arrival) developed clinical signs before inoculation and was eliminated from the study.

Approximately 1 mo following arrival, the controls were sham inoculated intranasally with 100 µl sterile SP4 broth in each naris, and tortoises in the naive and challenge groups were inoculated in each naris with 100 µl of SP4 broth containing approximately 10^4 colony forming units (CFU) of *M. agassizii* strain 723, for a total dose of 10^8 CFU. The 723 isolate was obtained originally from a clinically ill tortoise from Sanibel Island, Lee County, Florida. The sentinel tortoises were captured, but received no other treatment.

Following inoculation, observations of all tortoises were attempted daily to determine the onset and sequence of clinical signs. Behavior also was monitored. At 2 - 4 wk intervals post-inoculation (PI), tortoises were trapped, examined, and weighed, then tranquilized and blood, nasal flush and nasal swab samples were collected. Samples were processed as previously described. A total of 22 tortoises was examined at necropsy and histologically. Four control and nine naive tortoises were euthanatised and necropsied in October, 1994, before undergoing winter dormancy; seven challenge and two naive animals were euthanatised and necropsied in March, 1995, after emergence. Complete necropsies (n = 15) were performed on two control, six naive and seven challenge tortoises; only heads were examined on the other animals (n = 7).

Results

Clinical Signs

Of the six control group tortoises, none showed consistent clinical signs nor had positive culture, PCR, or ELISA results. At each of three time points, one control tortoise exhibited mild ocular signs that were probably associated with environmental conditions. One of two sentinels developed clinical signs at 12 wk PI, although she had not been inoculated with culture or medium. Of the naive tortoises, 67% exhibited clinical signs beginning 2-3 wk PI, 79% were clinically ill by 8 wk PI, and 94% had shown signs by 16 wk PI (Table 4-1). One naive tortoise never exhibited clinical signs. All seven challenged tortoises developed clinical signs of URTD before dormancy. Five (71%) challenged tortoises and one naive tortoise exhibited clinical signs soon after emergence. No tortoises became moribund or died during the study.

At 2 wk PI, six challenge and 17 naive tortoises were examined. Challenged tortoises had higher total clinical sign scores than naive tortoises (2.6 vs. 0.5, $P < 0.001$), and higher scores for nasal discharge (1.5 vs. 0.1, $P < 0.001$), ocular discharge (0.8 vs. 0, $P < 0.02$), and palpebral edema (1.5 vs. 0.6, $P < 0.02$) (Table 4-1). Significantly more challenge than naive tortoises exhibited nasal and ocular discharge (67 vs. 6%, $P < 0.01$; 50 vs. 0%, $P < 0.02$; respectively) (Figure 4-1). No consistent differences were seen between the two groups at later sampling times (Figure 4-2).

Table 4-1. Percentages of naive and challenge tortoises positive for each clinical sign of URTD, and mean clinical sign scores. Scores <1.5 are classified as slight, ≥1.5 and <2.5 as moderate, and ≥2.5 as severe. ND = nasal discharge, OD = ocular discharge, ED = palpebral edema, CJ = conjunctivitis. $P < 0.05$ indicates significant difference between groups.

Time	% Positive			Mean Score		
Sign	Naive	Challenge	*P*	Naive	Challenge	*P*
2 wk						
Total	67	83	0.626	0.5	2.6	0.000
ND[a]	6	67	0.007	0.1	1.5	0.000
OD	0	50	0.011	0	0.8	0.015
ED	59	83	0.369	0.6	1.5	0.012
CJ	53	83	0.340	0.6	1.0	0.345
4 wk						
Total	79	80	1.000	1.5	1.9	0.425
ND	50	60	1.000	0.8	1.2	0.254
OD	46	20	0.366	0.8	0.6	0.666
ED	52	40	1.000	0.7	0.8	0.864
CJ	59	60	1.000	1.0	0.8	0.670
6 wk						
Total	94	100	1.000	2.1	2.7	0.229
ND	67	83	0.626	1.1	1.9	0.040
OD	44	57	0.673	0.5	1.0	0.118
ED	72	71	1.000	1.0	0.9	0.652
CJ	89	43	0.032	1.4	0.7	0.055
8 wk						
Total	82	83	1.000	2.6	2.4	0.688
ND	71	71	1.000	1.4	1.6	0.677
OD	54	57	1.000	0.9	0.7	0.479
ED	71	71	1.000	1.1	1.3	0.634
CJ	71	29	0.075	1.5	0.6	0.004
12 wk						
Total	83	83	1.000	2.4	2.4	0.989
ND	62	71	0.642	1.5	1.6	0.836
OD	54	57	0.676	1.0	0.7	0.418
ED	67	71	0.667	0.7	1.3	0.068
CJ	79	29	0.198	1.1	0.6	0.105
Nx						
Total	84	83	1.000	2.6	2.4	0.744
ND	79	71	0.646	1.4	1.6	0.733
OD	79	57	0.340	1.2	0.7	0.123
ED	58	71	0.668	1.0	1.3	0.283
CJ	63	29	0.190	1.3	0.6	0.036

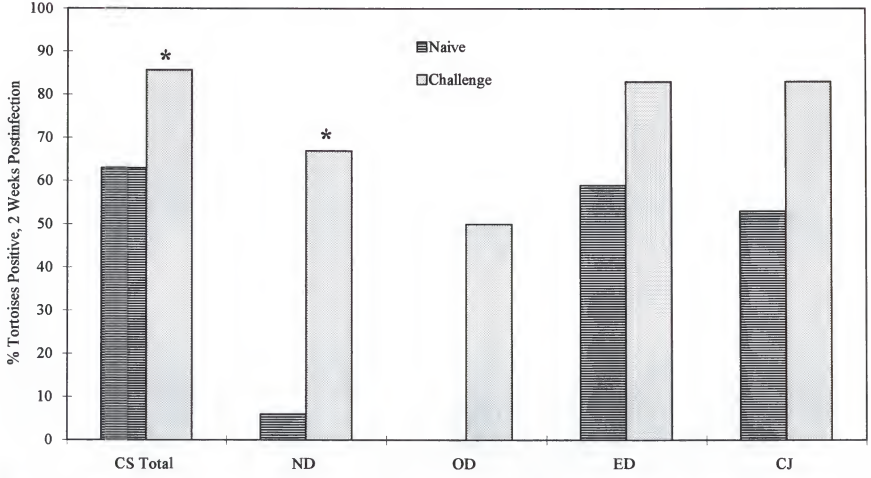

Figure 4-1. Percent of gopher tortoises infected with *Mycoplasma agassizii* positive for any clinical sign and each individual sign at 2 weeks postinfection. Tortoises in the challenge group had serological evidence of prior exposure to *M. agassizzi,* but those in the naive group did not. CS = clinical signs, ND = nasal discharge, OD = ocular discharge, ED = palpebral edema, CJ = conjunctivitis. * Indicates significant difference between groups, $P < 0.05$.

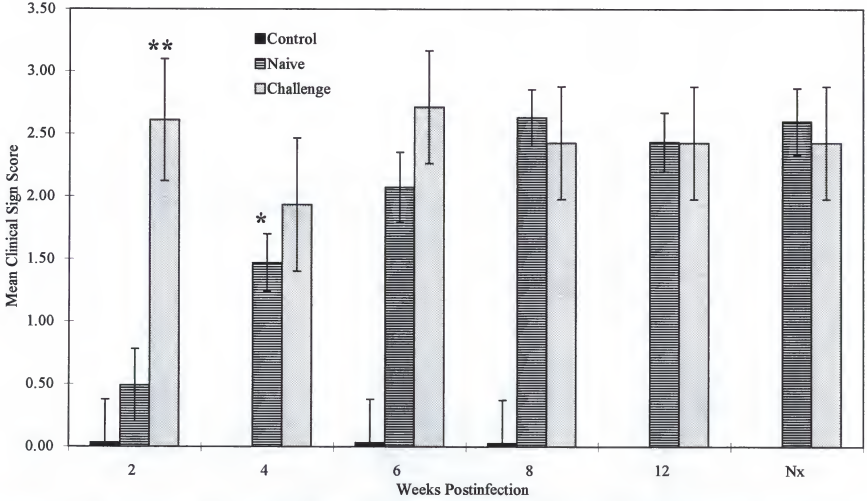

Figure 4-2. Total clinical sign scores for three groups of tortoises, one control and two experimentally infected with *Mycoplasma agassizii*. Bars represent standard errors. ** Indicates a significant ($P < 0.05$) rise in clinical sign score and a significant difference between groups. * Indicates a significant rise in clinical sign score.

Culture and PCR Results

The sentinel tortoise that became ill also had positive culture and PCR results. All inoculated tortoises had at least two positive culture results, and 17 of 18 had positive cultures from samples collected at necropsy. Mycoplasmal DNA was detected by PCR analysis from each inoculated tortoise, including those that did not exhibit clinical signs of URTD when sampled. By RFLP analysis, all isolates corresponded to *M. agassizii*.

ELISA Results

There was no anti-*M. agassizii* antibody response by the control tortoises to sham inoculation, although one sentinel had seroconverted by 4 wk PI. All inoculated tortoises seroconverted or had significantly increased ELISA values. ELISA values were greater for challenge than naive tortoises at each time point (Table 4-2, Figure 4-3). A significant increase in ELISA values of challenged tortoises was observed by 4 wk PI (mean ratio of samples to negative control of 6.3 vs. 3.3 at 0 wk, $P < 0.05$), and seroconversion of naive tortoises was observed by 6 wk PI (ratio of 3.9 vs. 1.5 at 0 wk, $P < 0.05$). The ratio of the challenge to the naive ELISAs increased at 2 and 4 wk PI, then declined.

Table 4-2. Least-squares mean ELISA values for naive and challenge tortoises. $P < 0.05$ indicates a significant difference between groups.

Time	Naive	Challenge	*P*
0 weeks pi	0.2049	0.4707	0.0437
2 weeks pi	0.2235	0.5925	0.0126
4 weeks pi	0.2439	0.8848	0.0001
6 weeks pi	0.5464	1.5254	0.0001
8 weeks pi	0.8314	1.8219	0.0001
12 weeks pi	1.4062	1.9839	0.0001
Necropsy	1.6796	2.2889	0.0001

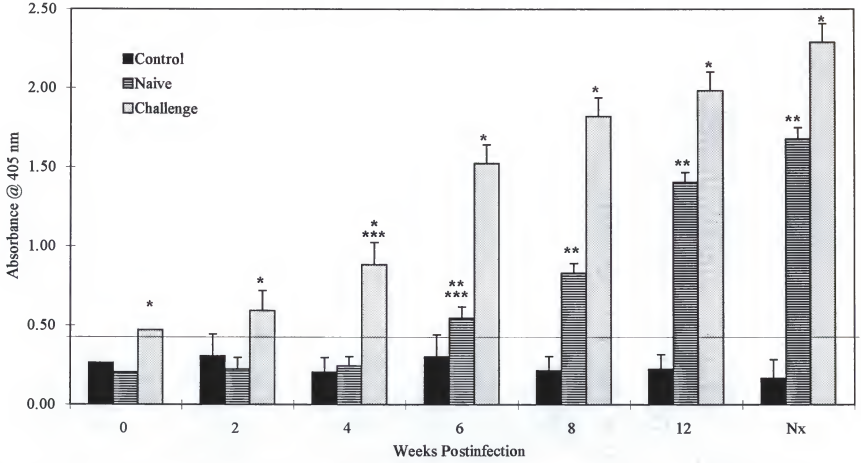

Figure 4-3. ELISA results for three groups of tortoises, one control and two experimentally infected with *Mycoplasma agassizii*. Tortoises in the naive group had no previous exposure to *M. agassizii*, while those in the challenge group had evidence of prior exposure. Line indicates ratio value (sample to negative control) of 3.0. * Indicates significant differences ($P < 0.05$) between challenge and naive groups. ** Indicates significant differences between naive and control groups. *** Indicates the first significant differences from Time 0 value for that group. Bars represent standard errors.

Histology Results

The upper respiratory tracts of the four control tortoises examined at necropsy had normal histologic appearances. In contrast, all tortoises inoculated with mycoplasma showed lesions similar to those seen in naturally occurring URTD. All challenge tortoises had moderate to severe inflammation and changes in the epithelium and submucosa. Three naive tortoises had minimal lesions and eight had moderate to severe abnormalities.. Lesions were consistently seen in the ventrolateral depression of the nasal cavity, a region immediately caudal to the vestibule (see Chapter 3).

Discussion

No tortoises inoculated with sterile medium developed clinical signs or histopathologic lesions, indicating that lesions were not a result of the mechanical effect of the medium on the tissues, nor of host inflammatory response to the medium. Experimentally infected naive animals did not seroconvert until 6 to 8 weeks PI. Because the sentinel that became ill and seroconverted was sampled only on arrival and 8 wk following arrival (4 wk PI for the inoculated tortoises), the timing of its seroconversion is uncertain. No known clinically ill tortoises were transported or held with the experimental animals once they left the Orange County holding facility, and the sentinel was not held in the facilities or with other tortoises during the time tortoises were being inoculated. Additionally, in an experiment involving the transfer of healthy tortoises into pens immediately following the removal of ill tortoises, there was no indication of environmental transfer, although the sample sizes were very small (see Chapter 7).

Therefore, the sentinel whose seroconversion was detected 8 wk after arrival probably was exposed to *M. agassizii* just before transport to UF.

Tortoises harboring *M. agassizii* may not show clinical signs, may exhibit mild signs, or may show signs only intermittently. Because tortoises not showing any ocular signs or nasal discharge sometimes have positive culture and PCR results, the organism may be transmissible from asymptomatic tortoises under appropriate conditions. Due to unavoidable constraints on sampling live animals, I cannot exclude the possibility that clinically healthy, seropositive, culture and PCR negative animals harbor the bacteria. As shown by the two tortoises (the sentinel and the tortoise that was eliminated from the study) that initially had negative culture and PCR results but developed disease without being inoculated experimentally, some infections may go through an extended latent period with low numbers of organisms in the nasal passages, or animals that appear to have cleared the organism may, in fact, have not.

The clinical response of challenged animals was more rapid and severe than that of naive animals, indicating that no protection was conferred by previous exposure to the organism. This is consistent with some other mycoplasmal diseases in which the immune response confers limited or no protection (Ellison et al. 1992), or contributes to pathogenesis, such as arthritis in fowl caused by *M. synoviae* (Kume et al. 1977), conjunctivitis in cattle caused by *M. bovoculi* (Rosenbusch 1987), and pneumonia in humans caused by *M. pneumoniae* (Krause and Taylor-Robinson 1992). Tully et al. (1995) have found that some mycoplasmal surface proteins share sequence and structural homologies with vertebrate proteins, and suggest that these may play a role in eliciting autoimmune responses. Repeated exposure to mycoplasmal proteins that resemble a

host's proteins may sensitize the host and induce an autoimmune response, leading to chronic manifestations of disease even if the primary etiologic agent is cleared.

Although actively produced antibodies apparently do not confer protection to adult tortoises, it is unknown if passively transferred antibodies (see Chapter 6) provide protection against URTD for hatchlings. Preliminary observations indicate that seronegative hatchlings are at least as susceptible to infection as adults, and that the disease progresses more rapidly, with high morbidity in the first 6 mo PI (see Chapter 5). Desert tortoise hatchlings exposed to infected adults developed clinical signs of URTD and suffered extremely high mortality rates (51 of 69, or 74%, of those with clinical signs died) (Oftedal et al. 1996). *Mycoplasma agassizii* was isolated from necropsied individuals, and severe inflammation was seen histologically. The immune status of the hatchlings and parents was unknown prior to the development of the outbreak, so no inferences can be drawn regarding the potential role of maternal antibodies in the course of URTD in hatchlings. Because cellular components probably play a major role in the pathogenesis of URTD and those are not transferred vertically in reptiles, the maternal antibodies may provide some protection. It would be interesting to explore the question of increased susceptibility to or severity of disease vs. protection from disease in hatchlings that have received maternal antibodies against *M. agassizii*.

The immune response stimulated by previous exposure to *M. agassizii* did not prevent reinfection or ameliorate signs of URTD after subsequent inoculation. The onset of clinical signs was, in fact, more rapid and the initial severity was greater than in first infections. Development of specific antibodies against *M. agassizii* did not ensure clearance of the organism by the tortoises, either on initial or subsequent exposure. Based

on this study, tortoises testing positive for antibodies against *M. agassizii* cannot be considered good candidates for release in repatriation, restocking, or relocation efforts. It may be acceptable to release seropositive tortoises in areas that already have seropositive animlas, as long as the overall prevalence is not increased significantly. Seropositive tortoises may provide desirable genetic material (see Chapter 6), and would be valuable as research subjects to study interactions with other disease agents and the effects of repeated exposure on metabolism, autoimmune responses, reproduction, and survival.

CHAPTER 5
HORIZONTAL TRANSMISSION OF *MYCOPLASMA AGASSIZII*

Introduction

In order to develop management and conservation plans that incorporate the potential of disease to affect populations, epidemiologic questions must be addressed. Of particular concern are the probability of transmission of the disease organism from one tortoise to another (horizontal transmission), the rate of spread within populations, and the potential for spread to nearby populations. The probability of transmission may be related to a tortoise's clinical and culture or PCR status. Infected tortoises may be culture and/or PCR positive without showing clinical signs. Conversely, it is sometimes difficult to detect bacteria in clinically ill tortoises. I designed an experiment to test the hypothesis that horizontal transmission of *M. agassizii* will occur only from those individuals that are clinically ill, and PCR and/or culture positive.

Methods

Acquisition of tortoises, intake and husbandry procedures, clinical assessments, sampling methods, ELISA and PCR procedures, and necropsy procedure were as detailed in Chapter 2.

Experimental Design

Fifteen pairs consisting of one male and one female were established in August, 1994. Due to the limited availability of seropositive and/or clinically ill tortoises, the sample design was not balanced. Five pairs of asymptomatic, ELISA-, PCR-, and culture-negative control tortoises were established as controls. The other ten pairs consisted of a clinically healthy, ELISA-, PCR-, and culture-negative tortoise that had been at UF since April, 1994 (resident), and an ELISA-positive or -suspect (irrespective of PCR or culture status) tortoise of the opposite sex and similar size (Figure 5-1, Table 5-1). One resident female was paired with an ELISA-negative, but clinically ill, culture- and PCR-positive male. The serosuspect and two seropositive tortoises were clinically healthy, and had negative culture and PCR results. The remaining six seropositive tortoises showed moderate clinical signs of illness. Three of those six were culture- and PCR-negative, one was culture- and PCR-positive, one was culture-positive but PCR-negative, and one was culture-negative and PCR-positive.

Behavioral observations and clinical signs of URTD were recorded opportunistically (see Chapter 2), and blood and nasal flush samples were collected from all tortoises in August and October, 1994, and in March and August, 1995. In 1996, samples were collected in February or March, and in July or August. Clinical signs and weights were recorded, and photographs were taken at each sampling time. Data were analysed using Chi-square and Fisher Exact tests for differences in proportions of tortoises becoming infected under different exposure conditions. Infected animals were euthanatized in July, 1996, except as noted below, and uninfected tortoises were released pursuant to Florida Game and Fresh Water Fish Commission permit number WR96224.

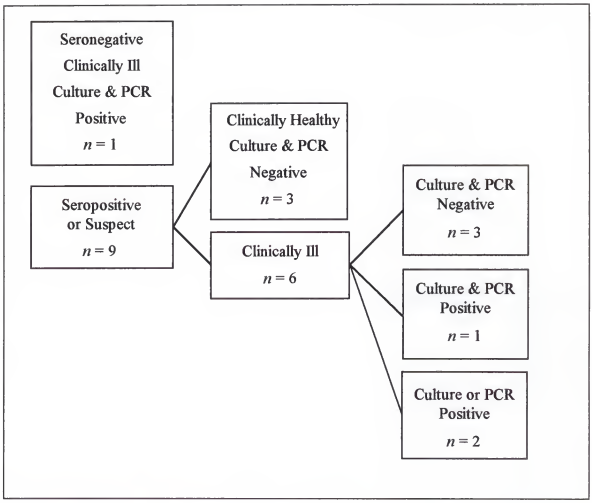

Figure 5-1. Flow chart showing initial distribution of seropositive, clinically ill, culture and PCR positive tortoises.

Table 5-1. Clinical signs, culture, PCR, and ELISA status of gopher tortoises included in the upper respiratory tract disease pairing study at each sampling time. CS - clinical signs (nasal or ocular discharge, palpebral edema, and/or conjunctivitis), Cl - culture results, P - PCR results, E - ELISA results.

Pen	ID	Aug. 1994				Oct. 1994				Mar. 1995				Aug. 1995				Feb.-Mar. 96				Jul.-Aug. 96			Necropsy
No.	Gender	CS	Cl	P	E	CS	Cl	P	E	CS	Cl	P	E	CS	Cl	P	E	CS	Cl	P	E	CS	P	E	Date
A1	226 F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R[a]
	201 M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
A2	577 F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	s[b]	7/96
	147 M	-	-	-	-	-	-	-	-	-	-	-	-	+[c]	+	+	-	+	-	-	+	+	-	+	7/96
B3	261 F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
	160 M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
B4	129 F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	H[d]
	211 M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
B5	108 F	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
	140 M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
D1	135 F	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	s	+	+	-	+	+	-	+	7/96
	241 M	-	-	-	+	-	-	-	+	-	-	-	+	+	+	+	+	+	+	-	+	+	-	+	7/96
D2	275 F	+	-	-	+	-	-	-	+	-	-	-	+	+	-	-	+	-	-	-	+	-	-	+	7/96
	144 M	-	-	-	-	-	-	-	-	-	-	-	-	disappeared											U[e]
D3	151 F	+	+	+	+	+	+	s	+	+	-	+	+	-	-	s	+	-	-	-	+	s	-	+	7/96
	126 M	-	-	-	-	+	+	+	+	+	-	+	+	+	+	-	+	+	-	-	+	-	-	+	7/96
D4	186 F	-	-	-	-	-	-	-	-	-	-	s	-	+	+	-	+	+	+	-	+	+	-	+	7/96
	235 M	+	+	-	+	-	+	-	+	+	-	-	+	-	-	+	+	-	-	-	+	s	-	+	7/96
D5	213 F	-	-	-	-	+	-	-	+	+	+	+	+	+	+	+	+	euthanatised[f]							6/95
	185 M	+	-	+	+	+	+	+	+	+	+	-	+	+	+	-	+	euthanatised[g]							6/95
D6	150 F	-	-	-	-	+	+	+	s	+	+	+	+	+	s	-	+	+	+	-	+	+	-	+	7/96
	183 M	+	+	+	-	+	+	-	+	+	+	+	+	+	+	-	+	disappeared							U[h]

Table 5-1--continued--

Pen	ID	Aug. 1994				Oct. 1994				Mar. 1995				Aug. 1995				Feb.-Mar. 96				Jul.-Aug. 96			Necropsy
No.	Gender	CS	Cl	P	E	CS	Cl	P	E	CS	Cl	P	E	CS	Cl	P	E	CS	Cl	P	E	CS	P	E	Date
D7	190 F	-	-	-	-	s	-	-	-	s	-	-	-	-	-	-	-	-	-	-	-	disappeared			U[a]
	228 M	-	-	-	+	-	-	-	+	-	-	-	+	-	-	-	+	s	-	-	+	+	-	+	7/96
D8	257 F	+	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+	+	-	+	+	-	+	7/96
	130 M	-	-	-	-	-	-	-	-	s	-	-	-	+	+	-	s	+	+	-	+	+	-	+	7/96
D9	205 F	-	-	-	s	+	-	-	-	?[j]	-	-	-	+	-	-	-	+	-	-	-	s	-	-	H
	110 M	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	R
D10	287 F	+	-	-	+	-	-	-	+	-	-	-	+	-	+		+	euthanatised[k]							6/95
	125 M	-	-	-	-	s	-	s	-	+	-	-	-	s	-	-	-	s	-	-	-	-	-	-	R

[a]Released pursuant to Florida Game and Fresh Water Fish Commission permit number WR96224.

[b]Denotes suspect status. Clinical signs: very mild, transient signs that could have been associated with environmental conditions or mechanical irritation. Culture: presumptive colony(ies) of *Mycoplasma* on agar that failed to grow when transferred to broth, or were overgrown by contamination before definite determination could be made. PCR: a very faint, nearly undetectable, signal. For ELISA, a ratio value between 2.0 and 3.0.

[c]Burrowed into an adjacent pen and was exposed to an experimentally infected tortoise.

[d]Hibernating as of December 1, 1996.

[e]Disappeared, presumed dead in burrow. Last examined August, 1995.

[f]Euthanatised due to uterine rupture.

[g]Euthanatised due to poor clinical prognosis.

[h]Disappeared, presumed dead in burrow. Last examined March, 1996.

[i]Disappeared, presumed dead in burrow. Last examined May, 1996.

[j]Unable to evaluate ocular signs prior to administration of ketamine.

[k]Euthanatised due to failure to reproduce and large mass viewed on radiography.

Results

General Observations

Observations indicated varying degrees of interaction between pair members. Several males were excluded from the original burrow in the pen by their female partners. Until one of the two dug a new burrow, those males, particularly 125, 185, 201, and 235, spent most of the day in a corner of the pen, under the shade cloth, and sometimes spent the night out of the burrow. Other pairs spent considerable time in courtship, or foraging in close proximity to one another.

In March, 1995, five hatchling tortoises were found in pen D1. The female (135) may have been gravid or storing sperm when she arrived at the UF facility in April 1994, and laid the clutch during the summer of 1994. Alternatively, she may have copulated with the male (241) and ovulated shortly after the pairing in August 1994. Based on gopher tortoise physiology (Taylor 1982), however, I believe the former explanation is more likely. One hatchling was removed and euthanatised due to congenital abnormalities; the other four were left in the pen.

Three tortoises were euthanatised in June 1995 (Table 5-1). One, number 287 (pen D10), was an initially seropositive, clinically ill, culture and PCR negative female whose partner (125) had not become ill. She failed to reproduce, and a large mass could be seen on radiographs. At necropsy, a large urolith (bladder stone), approximately 5 cm in diameter, was removed. The second, number 213 (pen D5), was an initially seropositive, clinically ill, culture and PCR positive female whose partner had become ill within one month of pairing. She failed to lay six eggs, and on exploratory surgery it was discovered that both oviducts had ruptured and the eggs were free in the coelomic cavity.

She was euthanatised without recovery from anesthesia. Her partner, number 185, was clinically ill, lethargic, and losing weight, and was euthanatised shortly thereafter.

One healthy, seronegative male tortoise (144, pen D2), paired with a seropositive female (275) disappeared after March 1995 (Table 5-1). He may have been overturned during interactions with the female and been pushed into the burrow, where he would have been unable to right himself. At the time of his disappearance, he had exhibited no indications that transmission of *M. agassizii* had occurred. The male tortoise from pen D6 (183) also disappeared, and may have met a fate similar to that of 144. His disappearance may have been related to agonistic interactions with any of the tortoises originally in pens D1, D6, D7, or D8, as discussed below. Due to the nature of tortoise burrows, it was impossible to determine unequivocally the fates of the animals that disappeared.

Approximately 10-11 months after pairing, in June and July 1995, the burrowing activities of the large number of tortoises in the small area resulted in the communication of several pens (Figure 5-2). Tortoises in pens D1, D6, D7, and D8 moved regularly between pens and had numerous agonistic encounters with one another. The four tortoises in pens D3 and D4 also moved between pens and had frequent interactions. Although tortoises from pens D9 and D10 also interacted with each other, aggressive interactions were not observed. One control male (147, pen A2), burrowed into an adjacent pen (A7) containing an experimentally infected ill tortoise, and moved between the two pens over several months. Neither the control female (577) nor the third tortoise was observed to switch pens. The study was originally designed to last for 12-14 mo, but when the animals began moving between pens, it was extended to 24 mo in order to collect more data.

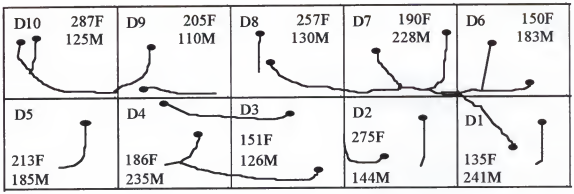

Figure 5-2. Burrow map showing interconnections among pens D1, D6, D7, and D8; between D3 and D4, and between D9 and D10. ● Burrow entrances.

Evidence of Transmission of *Mycoplasma agassizii*

The male control tortoise (147) became ill, seroconverted, and had positive culture and PCR results (Table 5-1). His partner became clinically ill and seroconverted after he returned to his original pen. No other control tortoises became ill or seroconverted. The D-section burrowing activities mentioned above resulted in the exposure of all tortoises in pens D1 and D7, which until that time had been clinically healthy, to clinically ill tortoises with positive culture and/or PCR results. Three became clinically ill, and had increased ELISA readings, with one of two initially negative tortoises seroconverting. Those activities also resulted in the exposure of the tortoises in pen D8 to clinically ill, culture and PCR positive tortoises. The seronegative male (130) became ill, had positive culture results, and seroconverted; the clinically ill, seropositive female (257), which had been culture and PCR negative until that time, also became culture positive. All four hatchlings in pen D1 became severely ill, seroconverted, and had positive culture results. All were euthanatised, and pure cultures of *M. agassizii* were recovered from the nasal cavities and conjunctiva on necropsy.

The initially seronegative, clinically ill, culture and PCR positive male (183) had seroconverted by October 1994. Based on his original samples from July 1994, and the ELISA values of his October samples, he probably seroconverted within 2 wk of pairing, and was considered as ELISA positive for the analyses (see below). In the ten experimental pairs, eight initially asymptomatic members, two of which were originally ELISA-positive and six of which were originally ELISA-negative, became moderately ill with signs of URTD and had increased ELISA values, with the latter six seroconverting. Two other tortoises (125 and 190) showed mild clinical signs that may have been associated with mechanical irritation or environmental conditions, but did not seroconvert.

Of the four initially uninfected tortoises that showed no increase in antibody levels, three did not have positive culture or PCR results. One (190) had only mild ocular signs, and two (110 and 144) never showed clinical signs. The fourth (125), which consistently showed mild signs and had a suspect PCR result in October 1994, was exposed to a tortoise (287) that had positive samples only at necropsy. Based on observations, they had only limited interactions. The serosuspect female (205), who was in contact with both 110 and 125, showed occasional, transient, mild clinical signs, but never had positive cultures or PCR results, nor did her antibody levels increase. In fact at all sample times after August 1994, she was classified as seronegative.

Of the 16 originally culture and PCR negative tortoises, 10 later had positive or suspect culture or PCR results. Eight were exposed to clinically ill, culture or PCR positive tortoises, seroconverted and had positive results, the ninth had positive culture results only from necropsy samples, and the last was the individual (125) discussed above. No tortoise became ill unless exposed to another ill tortoise.

Transmission Probabilities

Analysis of the data was complicated by the changing exposure status of the tortoises due to the housing problems discussed above. Therefore, analyses were carried out on three data sets. The first consisted of data from the first 10 mo (August 1994 - June 1995) of the study, the second from the last 14 mo (June 1995 - July 1996), and the third of the cumulative data. The first data set (Table 5-2) consisted of the original 15 pairs. The second set (Table 5-3) did not include those pairs in which transmission had already occurred, but only the control pairs and those whose exposure status had changed, leaving 11 observations. The third set (Table 5-4), with 22 observations, included some tortoises twice, as the observations of interest were the exposure events themselves, and not the pairs or individual tortoises. Although sample sizes were very small, and some cells had no observations, chi-squared tests of differences in proportions were run on the three data sets. The final set was collapsed on the three predictor variables (ELISA, clinical, and culture/PCR status; Table 5-5) to determine which had the most influence on transmission probability.

Four exposure status classes were established based on ELISA classification, clinical signs, and culture or PCR status. Class assignment was based on a tortoise's status at any point during the time period of interest. Therefore, a tortoise's status could change between the first and last parts of the study. Each criteria was recorded as positive or negative, and transmission was recorded as yes or no. Transmission was classified very conservatively, with any tortoise exhibiting even mild, transient, clinical signs not obviously related to mechanical irritation (e.g., plant material in the eye or nose) or environmental conditions (e.g., being held overnight in a box in which the tortoise had

urinated), or having one suspect culture or PCR result recorded as having been infected by horizontal transmission. The four classes were 1) ELISA, clinically, and culture/PCR negative (-/-/-), 2) ELISA positive, clinically and culture/PCR negative (+/-/-), 3) ELISA and clinically positive, and culture/PCR negative (+/+/-), and 4) ELISA, clinically, and culture/PCR positive (+/+/+). The first data set (Table 5-2) consisted of the following observations (class, yes, no): 1,0,5; 2,0,2; 3,2,2; and 4,4,0. The second set (Table 5-3), for the last 14 mo, was comprised of the following observations: 1,0,4; 2,0,1; 3,0,1; and 4,5,0. The last, cumulative, data set, included the following observations: 1,0,5; 2,0,3; 3,2,3; and 4,9,0. Chi-square tests of association of exposure status and transmission were significant for all data sets, with 3 degrees of freedom (df) and $P < 0.001$ for all sets (Tables 5-2, 5-3, 5-4).

Table 5-2. Contingency table for exposure status and transmission occurrence for the first 10 mo (August 1994 - June 1995) of the gopher tortoise URTD pairing study.

Exposure Status ELISA/Clinical/Culture or PCR	Transmission Yes	No
-/-/-	0	5
+/-/-	0	2
+/+/-	2	2
+/+/+	4	0
χ^2, 3 df = 10.8, $P = 0.013$, power = 0.80		

Table 5-3. Contingency table for exposure status and transmission occurrence for the last 14 mo (June 1995 - July 1996) of the gopher tortoise URTD pairing study.

Exposure Status ELISA/Clinical/Culture or PCR	Transmission Yes	No
-/-/-	0	4
+/-/-	0	1
+/+/-	0	1
+/+/+	5	0
χ^2, 3 df = 11.0, $P = 0.012$, power = 0.81		

Table 5-4. Contingency table for exposure status and transmission occurrence for the duration (August 1994 - June 1995) of the gopher tortoise URTD pairing study.

Exposure Status ELISA/Clinical/Culture or PCR	Transmission Yes	No
-/-/-	0	5
+/-/-	0	3
+/+/-	2	3
+/+/+	9	0
χ^2, 3 df = 17.2, P = 0.0006, power = 0.96		

Table 5-5. Transmission probabilities and tests of significant differences of proportions for different exposure status classes of tortoises relative to upper respiratory tract disease.

Exposure Status	Transmission Yes	No	Probability of Transmission	Fisher Exact Test, P value
ELISA				
negative	0	5	0	
positive	11	6	0.65	0.018
Clinical illness				
negative	0	8	0	
positive	11	3	0.79	0.001
Culture / PCR				
negative	2	11	0.15	
positive	9	0	1.00	0.0002

Because transmission was defined very conservatively for the purposes of this study, two tortoises were categorized as having been infected via horizontal transmission based solely on the appearance of mild clinical signs. Neither animal seroconverted nor had positive or suspect culture or PCR results during the second half of the study. Both tortoises were housed with clinically ill, seropositive tortoises that were culture and PCR negative. If those tortoises were designated negative relative to transmission, the final

data set was comprised of the following observations: 1,0,5; 2,0,3; 3,0,5; and 4,9,0. The chi-squared value was 22.0, with 3 df and $P < 0.0001$.

When Table 5-4 was collapsed on ELISA status (Table 5-5), none of the tortoises in five ELISA negative pairs became ill or seroconverted, but of the 17 interactions involving ELISA positive tortoises, 11 resulted in transmission, for a probability of 0.65. Fisher Exact test of a difference in proportions was significant at $P = 0.018$. When collapsed on clinical status, none of eight interactions involving negative tortoises resulted in transmission, as defined above, while 11 of 14 (probability 0.79) involving clinically ill tortoises did (Fisher exact test, $P = 0.001$). If transmission was defined more liberally, and designated as positive only if a tortoise seroconverted and had clearly positive culture or PCR results, then nine of 14 (probability 0.64) interactions with clinically ill tortoises resulted in transmission (Fisher exact test, $P < 0.006$). When transmission was defined conservatively, for culture and/or PCR status, 2 of 13 culture/PCR negative class interactions resulted in transmission (probability 0.15), while all of nine positive class pairings effected transmission (Fisher exact test, $P = 0.0002$). If transmission was defined more liberally, then none of 13 culture/PCR negative interactions resulted in transmission, and the Fisher exact test was significant with $P = 2 \times 10^{-6}$.

Discussion

The unplanned movements of the tortoises resulted in more exposure events than initially planned, and possibly compromised some of the original experimental design. However, at least one pair remained in each exposure category and was followed for 24

mo, twice as long as originally planned. Therefore, the intent of the study was not compromised by the tortoises' natural behavior.

Although manifestation of latent infections could not be excluded absolutely, the results supported the hypothesis that *M. agassizii* was horizontally transmitted between adult tortoises, and between adult and hatchling tortoises. The route of transmission may have been direct contact, aerosol, or fomite transmission. Direct contact was the most likely route. If aerosol transmission occurred, it was probably over short distances, such as would be likely to lead to direct contact. Sentinel and control animals housed in pen groups that also housed ill tortoises did not become ill or seroconvert, indicating that aerosolized bacteria were unlikely to travel even relatively short differences or over low (0.5 m) barriers. Similarly, fomite transmission is unlikely to play a major role (see Chapter 7). However, because many tortoise interactions occur in burrows, it is difficult to assess the importance of the various modes.

Although sample sizes were small, results supported the hypothesis that transmission is more likely when the "donor" is symptomatic, although tortoises without clinical signs may be infected and able to transmit the pathogen under appropriate conditions. After rainstorms, it is not uncommon to find two or more tortoises drinking from the same puddle (R. E. Ashton, pers. comm.; G.S.McLaughlin, pers. obs.). When tortoises drink, they often get water in their noses, which they then blow or "sneeze" out. An asymptomatic tortoise harboring bacteria may shed enough in this manner to infect a nearby conspecific via the aerosol route, or to leave an infective dose in the water or on nearby plants.

A concurrent study (Brown et al. 1996b), in which gopher tortoises were experimentally infected with varying doses of *M. agassizii*, demonstrated that the infective dose of the 723 strain is very low, less than 10 CFU. However, an additional isolate failed to induce disease or elicit an antibody response. In previous research involving desert tortoises, Brown et al. (1994) demonstrated that exudate from ill tortoises elicited a stronger immune response and induced more severe disease in inoculated tortoises than pure cultures of *M. agassizii*. In addition to the mycoplasma, the exudate probably contains other species of bacteria (e.g., *Pasteurella testudinis*, *Corynebacterium* sp.), as well as cellular components and cytokines that may elicit an immune response and contribute to the disease process.

The two tortoises that exhibited mild signs without seroconverting or having positive culture and PCR results may have been responding to cellular or chemical components in the exudate from their partners, to environmetnal conditions, or to other species or strains of bacteria that were only mildly pathogenic. Because conjunctival swabs were not collected from these animals, and aerobic cultures were not performed, those possibilities were not addressed in this study.

Further research regarding transmission probabilities and modes is needed. Larger sample sizes and balanced designs would allow more rigorous statistical analyses. However, establishing and maintaining such designs would be difficult, as tortoises' clinical, culture, and PCR status can vary over both the short and long term (e.g., see Chapter 4). Experiments entailing differences in pen design could be planned to estimate the probabilities of transmission by the various routes. For example, double layers of wire screening between pens, with sufficient distance between the layers to prohibit direct

contact, could be used to assess the probability of aerosol transmission. Pen designs in which water containers are shared, but direct contact and aerial transmission are precluded could generate data addressing the role of transmission via water. Tortoises' strength, determination, and digging abilities must all be considered when designing such experiments.

Seropositive, clinically healthy, culture and PCR negative individuals may be suitable for relocation, restocking, and repatriation programs, but clinically ill animals should not be used in such efforts. However, the proportion of tortoises that appear to have recovered from disease and eliminated the mycoplasma, while actually harboring the organism, is unknown. As evidenced in the preceding chapter, some of those tortoises may have latent infections that will recrudesce under stressful conditions.

CHAPTER 6
VERTICAL TRANSMISSION OF *MYCOPLASMA AGASSIZII*

Introduction

Many mycoplasmas can be transferred vertically, that is, from mother to offspring *in utero* or *in ovo* (see Chapter 1). Before appropriate decisions can be made concerning the fate of desert or gopher tortoises infected with *M. agassizii*, particularly those from threatened or endangered populations, questions regarding the vertical transmissibility of *M. agassizii* need to be resolved. If *M. agassizii* is not transmissible via eggs, the options for preserving genetic material are increased. I designed experiments to test the hypotheses that *M. agassizii* is transferred *in ovo* to gopher tortoise hatchlings and that levels of specific antibodies against *M. agassizii* in egg yolk and hatchling plasma are associated with those found in maternal plasma.

Methods

Adult tortoises were housed as detailed in Chapter 2, and selected as specified in Chapter 5. In order to obtain clutches of eggs, pairs were established in August, 1994, as detailed in Chapter 5. The sampling schedule was as detailed in Chapter 5.

Egg Collection and Incubation

Egg development was monitored by radiography (6 mas, 62-76 kv) beginning in April of 1995 and 1996. When eggshells were judged to be calcified adequately, oviposition was induced by intravenous injection of arginine vasotocin (Sigma) at approximately 10-12 picograms/kg. Tortoises were monitored until clutch deposition was complete or for a minimum of 4 hr. Eggs were collected as they were laid and placed in a sanitized container (washed with 1:10 bleach solution and air-dried upside down) partially filled with sterilized vermiculite moistened with an equal weight of sterilized water. Female identification number and letter indicating order of deposition were written on each egg with a graphite pencil swabbed with 70% ethanol. After approximately half the clutch was laid, cloacal swabs for mycoplasma culture were taken, streaked onto SP4 agar, placed in SP4 broth, and incubated as described previously.

In 1995, eggs were incubated at 29°C until hatching. In 1996, eggs were incubated at 27, 29 or 31°C. Approximately 1 wk before hatching each egg was cleansed of adhering vermiculite with clean gauze and placed into a sanitized plastic container. After hatching, resorption of yolk, and closing of the umbilicus, hatchlings were placed in containers with clean sand. Hatchlings were maintained separately from adults, at ambient temperature and light cycles, and fed natural foods supplemented with commercially available vegetables until release into outdoor pens, where they were fed natural foods.

Culture and PCR Procedures

Eggs were taken at various times during incubation for mycoplasma culture and antibody detection. At pipping, chorioallantoic-amniotic fluid (CAF) was collected and

frozen for later analyses. For culture, 100 µl samples of yolk and albumin, and small pieces of membrane were added to 900 µl of SP4 broth. After 48 hr incubation at 30°C, 500 µl of culture were removed for PCR analysis. All CAF samples obtained at pipping were treated in the same manner. In 1996, one or two pooled samples consisting of 100 µl of CAF from each of 3 - 6 eggs, mixed well, were made for each clutch and processed as described.

ELISA Procedures

Based on preliminary experiments using several methods of antibody extraction, the supernatant resulting from mixing 1 ml of yolk with 1 ml PBS-AZ provided the most efficient fraction for detection of antibodies (G. S. McLaughlin and I. M. Schumacher, unpub. data). Blood samples for ELISA were collected by cardiocentesis from most hatchlings at approximately 2-4 wk of age. In 1995, the goal was to obtain a blood sample from each hatchling. In 1996, sampling effort was reduced to a maximum of five samples per clutch due to the low coefficients of variation for the 1995 samples.

In 1995, hatchling ELISA samples were run as previously described except that three dilutions from the range 1:1 to 1:8 were run in duplicate, depending on the sample volume obtained. Sample dilutions were based on previous experiments with plasma from desert tortoise hatchlings (I. M. Schumacher, unpub. data). All hatchling or egg samples were run on the same plate as 1994 and 1995 samples from the corresponding female and the presumed sire, if possible. If samples were split between two plates, plates were run on the same day with the same reagents and positive and negative controls. In 1996, samples were run in triplicate at a dilution of 1:2, as that was the dilution used for analysis

of the 1995 samples. Additionally, that modification reduced the necessary blood volume from approximately 400 µl to 150 µl, reducing stress to the hatchlings. Samples collected in 1995 and 1996 from the corresponding female were run on the same plate as samples from their eggs and/or hatchlings.

Results

Clutch Sizes, Fertility and Hatching Rates

Twenty-six clutches, 13 each year, were collected. Arginine vasotocin was used to induce oviposition of 20 clutches and the remaining six clutches (five in 1995 and one in 1996) were laid in the pens. In 1995, 13 tortoises developed 115 eggs (mean = 8.8), and 103 eggs were recovered (Table 6-1). Five females laid entire clutches in the pens, four of which were removed within 1 wk of laying. One female laid nine eggs in an inaccessible area of her pen. Two females that laid incomplete clutches on induction laid the remaining eggs ($n = 3$) in their pens. Twenty-five eggs were removed for culture, PCR, and ELISA samples. Of those, five were fertile and removed while viable, four had died, and five were infertile. I could not determine fertility on the remaining 11.

Overall clutch sizes (Table 6-1) were larger in 1996 (mean = 11.1) than in 1995 (mean = 8.8, 2-tail t-test = 2.78, 17 df, $P = 0.013$), as were those of seronegative females (11.7 vs 9.8, 2-tail t-test = 2.45, 9 df, $P = 0.037$). In 1996, clutch sizes of seronegative females (mean = 11.7) were larger than those of seropositive females (mean = 10.3, 2-tail t-test = 2.30, 11 df, $P = 0.042$). Thirteen females developed 144 eggs, 130 of which were recovered. One female laid 11 eggs that were incompletely calcified and broke during the

laying process. Five eggs were laid in the pen and six while in transport; a usable sample for culture and PCR was obtained from only one egg. Three females retained one egg each, the fates of which were unknown. One fertile egg was donated for the purpose of establishing embryonic tortoise cell lines, and 19 eggs were sampled. Of those, 13 eggs were infertile, four had died during incubation, and I could not determine fertility of two.

In 1995, 78 eggs pipped and 77 hatchlings were produced in the laboratory (Table 6-1), and nine hatchlings were recovered from the 12 eggs laid in the pens (1/1, 1/2, 7/9). The tortoise that pipped but did not hatch had an abnormal yolk sac; it was either malformed or had ruptured, and yolk was found throughout the coelom. The hatchling was in respiratory distress (cyanotic and mouth breathing), and was euthanatised. Another fetus appeared to be full term, but had spina bifida in the cervical region, and only one kidney.

In 1996, 111 eggs produced 113 viable hatchlings (Table 6-1). One egg produced one healthy hatchling and one that was anencephalic. Two other eggs, from a different clutch, each produced healthy twins. There were no differences in fertility or hatching rates between infected and uninfected females for either year individually or both years combined (χ^2 tests, 1 df, all $P > 0.75$). Fertility and hatching rates did not differ significantly between years (χ^2 tests, 1 df, all $P > 0.20$).

Culture and PCR Results

No cultures or PCR assays of cloacal samples, egg yolk, albumin, or membranes were positive for mycoplasma, and no hatchlings isolated from adults developed clinical signs (see Chapter 5 for evidence of horizontal transmission from adults to hatchlings).

Table 6-1. Clutch data from gopher tortoise upper respiratory tract disease vertical transmission study, 1995 and 1996.

	1995			1996		
Serostatus	negative	positive	total	negative	positive	total
No. clutches	8	5	13	7	6	13
No. eggs	78	37	115	82	62	144
Mean	9.8[a]	7.4	8.8[a]	11.7[a,b]	10.3[b]	11.1[a]
No. recovered	75	28	103	69	61	130
No. sampled	16	9	25	11	8	19
Fertility rates	94%	97%	95%	88%	92%	90%
No. hatchlings	61	26	87	52	61	113
Hatching rates	90%	93%	91%	86%	88%	87%
CAF samples	59	19	78	51	51	102
Blood samples	51	25	76	29	28	57

[a]Clutch sizes differed significantly between 1995 and 1996 for seronegative tortoises (2-tail t-test = 2.45, $P = 0.037$), and for overall clutch size (2-tail t-test = 2.78, $P = 0.013$).

[b]Clutch sizes differed significantly between seronegative and seropositive tortoises for 1996 (2-tail t-test = 2.30, $P = 0.042$).

ELISA Results

For ELISAs in 1995, 76 hatchlings and 34 yolks were sampled for a total of 96% of all available eggs and hatchlings sampled. Total sampling effort per clutch ranged from 75-100%, with 50-100% of hatchlings sampled. In 1996, 57 hatchlings and 19 yolks were sampled, for a total sampling effort of 58%, with sampling effort per clutch of 42-100%. ELISA values in egg yolks and hatchlings were correlated with maternal ELISA values in Autumn of the prior calendar year ($r = 0.68$, $R^2 = 0.461$, $P < 0.0002$, $n = 25$; Figure 6-1), indicating that specific antibodies were transferred via the egg yolks to the hatchlings.

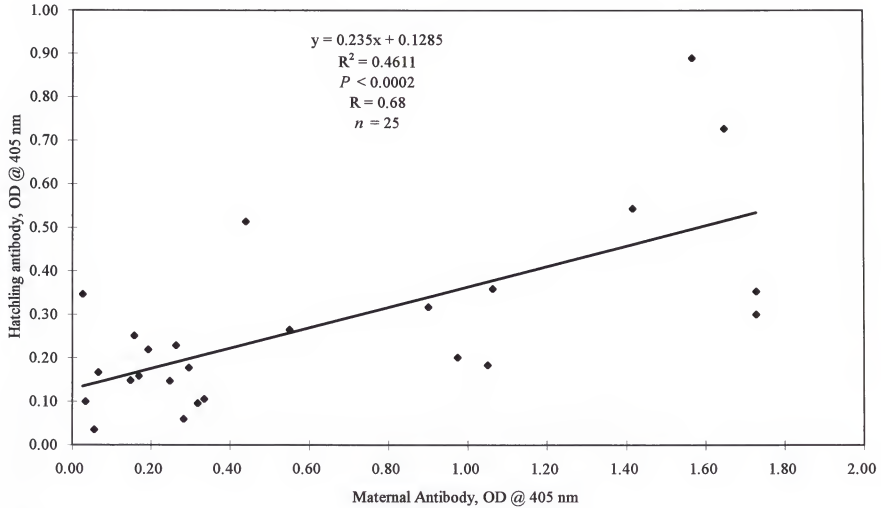

Figure 6-1. Regression of specific anti-*Mycoplasma agassizii* antibody levels of gopher tortoise hatchlings on maternal antibody levels, as measured by ELISA. OD = optical density.

Discussion

Unlike various mycoplasmal infections of rodents and poultry (Simecka et al. 1992) and wild ducks (Goldberg et al. 1995), there was no evidence to support a hypothesis of vertical transmission of *M. agassizii*. Therefore, it should be possible to collect eggs from infected female tortoises, incubate the eggs, and release the hatchlings with no risk of spreading the disease.

Vitellogenesis begins in August or September and continues to December (Taylor 1982), providing an extensive period for deposition of antibodies. In spite of that, the antibody level in egg yolks and hatchling plasma was only 10 -20% of that in the maternal plasma. Thus, there is no evidence that female gopher tortoises are sequestering antibodies in the egg components. This is in contrast to birds, particularly chickens, which deposit large amounts of antibodies in their eggs in a very short time period. Antibody levels in chicken eggs can be several orders of magnitude greater than that in the maternal plasma.

Hatchling antibody levels decline slowly over the first year of life, as the maternal antibodies are broken down (I. M. Schumacher, unpub. data). This process is much slower in tortoises than in mammals and birds, where such passively acquired antibodies decline within weeks or a few months after birth or hatching. It is not known if antibodies against *M. agassizii* affect juvenile responses to infection with the organism. Although adult tortoises that have developed an antibody response against *M. agassizii* respond very quickly and adversely to subsequent exposure (Chapter 4), that response may be mediated more by cellular immune components (e.g., macrophages and heterophils) than by

humoral. Hatchling and juvenile tortoises with maternal antibodies may be protected from some effects of infection if the antibodies are neutralizing in nature, and if they can be mobilized appropriately against the mycoplasma. Research needs to be conducted to address these areas.

CHAPTER 7
ENVIRONMENTAL TRANSMISSION OF *MYCOPLASMA AGASSIZII*

Introduction

Some mycoplasmas can remain viable for at least several days in the environment (Chandiramani et al. 1966), and for many years under refrigeration or freezing (Yoder and Hofstad 1964). One method of controlling mycoplasma infections in domestic stock is to depopulate a farm or facility, spray buildings and equipment with disinfectants, wait an appropriate amount of time, and then reintroduce uninfected stock (Anonymous 1989). In order to determine appropriate time frames for restocking tortoises, knowing the length of time *Mycoplasma agassizii* remains viable in the environment is critical. Unfortunately, direct viability testing is extremely difficult and impractical, if not impossible. Many soil bacteria and fungi in the tortoise burrow and surrounding environment grow very rapidly in culture and quickly overtake any mycoplasma colonies that might be present. However, the risk of environmental transmission of *M. agassizii* is an important parameter in the decision making process. In order to address this question, I designed an experiment to test the hypothesis that environmental transmission of *M. agassizii* would occur in tortoises introduced to pens previously occupied by infected, clinically ill tortoises.

Methods

As a result of previous and concurrent experiments, 13 seropositive tortoises infected with *M. agassizii* and 15 seronegative tortoises with no clinical signs and negative culture and PCR results were available. The 13 positive tortoises were captured and re-inoculated with approximately 10^8 colony forming units (CFU) of *M. agassizii* to ensure active clinical disease and shedding of bacteria at the time scheduled for introducing healthy tortoises to the pens (see Chapter 4). Six weeks following inoculation, the 13 tortoises were captured, as were the 15 healthy tortoises. Nasal flush samples were collected from each infected tortoise to analyze by PCR.

Immediately after capture and removal of five infected tortoises from their pens, a healthy tortoise was put into the pen. Transfers were carried out within 15 min. Transfers were also carried out at 3 ($n = 4$), 7 ($n = 3$), and 10 ($n = 3$) days following removal of the infected tortoises. Because only 13 ill tortoise pens were available, one of the day 7 and one of the day 10 transfers consisted of pairs of tortoises. Eight weeks following transfer, the 15 tortoises were captured, assessed clinically, photographed, and blood and nasal flush samples were collected. Samples were handled as previously described.

Results

All infected tortoises were clinically ill at the time of capture, although PCR results for all animals were negative. Transferred tortoises explored their new surroundings and usually entered the burrows within 1 hr, and some entered within 15 min. At 8 wk post-transfer, no transfer tortoise was clinically ill, or had positive culture or PCR results. No tortoise showed an increase in ELISA values indicative of possible infection (Table 7.1).

Table 7.1. ELISA results from initially clinically healthy, culture and PCR negative tortoises transferred into pens previously occupied by clinically ill, culture or PCR positive tortoises. Ratios for equal ELISA values may differ due to plate--to--plate variation in the assay.

Tortoise	Pre-transfer ELISA		Post-transfer ELISA		Transfer
ID	Value	Ratio	Value	Ratio	Day
108	0.275	1.89	0.242	1.66	0
129	0.164	1.17	0.137	0.98	0
160	0.185	1.75	0.236	1.80	0
201	0.217	1.70	0.192	1.51	0
205	0.471	3.37	0.409	2.93	0
110	0.119	0.94	0.137	1.08	3
211	0.222	2.00	0.234	2.11	3
219	0.352	2.72	0.184	1.43	3
311	0.134	1.21	0.144	1.29	3
159	0.278	2.12	0.160	1.23	7
226	0.279	2.63	0.212	2.00	7
261	0.378	2.92	0.287	2.21	7
119	0.113	1.02	0.120	1.08	10
125	0.063	0.60	0.054	0.52	10
140	0.134	1.23	0.152	1.40	10

Discussion

Although the PCR results for the re-infected tortoises were negative at the time of capture for transfer, the PCR is limited in its sensitivity. The lower limit of detection is approximately 1000 CFU (D. R. Brown, unpub. data), whereas the infectious dose of *M. agassizii* may be 10 or fewer CFU (Brown et al. 1996b). Therefore, tortoises could have been shedding infective doses of bacteria and still have negative PCR results.

In other studies, 100% of 28 naive tortoises seroconverted by 8 wk PI, with all tortoises tested at 6 wk PI having significantly increased antibody levels (Brown, D. R., 1996b; Schumacher, unpub. data; Chapter 4). No tortoises in this study had increased

antibody levels, and it is unlikely that insufficient time had passed for the tortoises to develop an immune response.

Environmental transmission of *M. gallisepticum* is of considerable concern to the poultry industry, necessitating disinfection of premises and equipment, and a 2-6 wk fallow period before introducing new stock (Anonymous 1989, M. B. Brown pers. comm.). The *M. gallisepticum* strain causing conjunctivitis in house finches also can survive in the environment and cause infections in individuals later housed in the same facility (Ley et al. 1996, Luttrell et al. 1996).

Environmental transmission of *M. agassizii* in the wild may not be of great concern. However, equipment used for capturing, handling, holding, and transporting tortoises should be cleaned after each use by spraying or wiping with bleach, ethanol or other disinfectant solution. Care should be taken to dispose properly of all gloves or drapes that may have become contaminated. Clinically ill tortoises should not be housed in direct contact with other animals, nor should indirect contact be allowed. Infected tortoises should not be able to sneeze on other tortoises, nor should water or food dishes be shared between pens without disinfection. Because the sample sizes at each time point were very small, further research, with more rigorous attempts to isolate mycoplasmas and quantify shedding, needs to be conducted. For the purposes of relocation, restocking, or repatriation efforts, a short fallow period, perhaps 2 wk, is probably sufficient to prevent environmental transmission of *M. agassizii*.

CHAPTER 8
CONSERVATION AND MANAGEMENT IMPLICATIONS OF UPPER RESPIRATORY TRACT DISEASE

The research reported in this dissertation has built on and extended the findings of research on upper respiratory tract disease in desert and gopher tortoises. Based on the knowledge that *Mycoplasma agassizii* causes clinical signs and lesions of URTD and elicits an antibody response detectable by an ELISA, experiments were designed to compare the pathological effects of the disease in gopher tortoises to those in desert tortoises, investigate secondary immune responses, and determine routes of transmission. The knowledge gained can be applied to conservation and management decisions, and new areas of research have been suggested.

Implications for Conservation and Management

When tortoises are impacted by development, mining, agriculture, or forestry practices, decisions must be made regarding their disposition. Tortoises can be ignored, temporarily removed and confined to pens for later return to the site, permanently moved to a currently inhabited site, moved to a formerly occupied site, donated to research or educational facilities, or euthanatised. The choices open for a particular population of tortoises depend on the location, historical, current, and future site use, surrounding land

use patterns, importance of the population in maintaining genetic variability, and political and social factors. No one prescription will be ideal for all situations, and it is difficult, if not impossible, to develop a set of prescriptions that will cover all permutations of the above factors. However, guidelines for making decisions can be developed, and some are presented in this chapter.

Establishing Goals

The goals of the management action(s) must be established before decisions can be made regarding what data to collect, or how to design survey or monitoring programs. Management tactics designed to establish, create, or maintain a URTD-free population will differ from those intended to maintain the status quo relative to disease agents. If the genetic material represented by tortoises in defined or isolated populations (e.g., those in South Carolina, Mississippi, or Louisiana, and some in Alabama) is important for conservation purposes, aggressive efforts to protect and maintain the gene pool may be of primary concern.

Understanding URTD and Test Results

An understanding of test results is necessary for proper interpretation and application. For field personnel, recognition of clinical signs of URTD is an important skill to gain. Differentiation of signs of URTD from other conditions is difficult even for experts, as the signs are nonspecific, but some conditions can be recognized fairly easily (e.g., foreign body in or trauma to the eye causing discharge, or nasal "discharge" due to

drinking or eating). Tortoise behavior interacts with clinical disease to affect transmission probabilities, so understanding behavior also is important in the decision making process.

A clinically healthy tortoise, with negative ELISA, culture, and PCR results is probably free from URTD. A positive ELISA, in the absence of clinical illness and positive culture or PCR results, indicates only that the tortoise has been exposed to *M. agassizi*. Because clinical signs and culture and PCR results can vary over time, we cannot predict if or when a seropositive tortoise will break with clinical disease and begin shedding bacteria. The more stress to which an animal is subject, such as human intrusion, handling or transport, drought or other extreme weather conditions, the more likely it is to have a disease recurrence. Repeated exposures to *M. agassizii* elicit more intense immunological responses by the tortoises, potentially leading to autoimmune responses that may contribute to the more severe lesions seen in longer-term infections, as well as possible liver pathology. Tortoises with rapid responses to the mycoplasma, with copious mucus production, may be more likely to transmit the agent to conspecifics, as their energy reserves have not been depleted by the disease process.

A clinically ill tortoise with positive culture or PCR results, regardless of ELISA results, probably is capable of transmitting *M. agassizii*. The more active a tortoise is, and the greater its daily movements, the more likely it is to spread the bacteria through a colony and foment an outbreak of URTD. Behaviorally, male tortoises have larger home ranges and more intraspecific contacts, so are probably at greater risk of coming in contact with and spreading the pathogen.

A clinically healthy tortoise (i.e., one with no nasal or ocular discharge or other signs) may have positive culture and PCR results, with either positive or negative ELISA results. It may be in the early stages of infection or recrudescence. In the former case, the ELISA value may be negative, but should rise within 6 wk. In the latter case, the ELISA value may be quite high. Such tortoises may be capable of transmitting the mycoplasma under the appropriate conditions. Although direct contact (nose-to-nose) seems to be the most important route, transmission through water or on food cannot be ruled out. When tortoises drink, they often expel water through their noses for short distances, up to 50 cm, or sneeze forcefully after drinking, aerosolizing the contents of the nasal passages. If tortoises are in close contact with one another, spatially or temporally, such occurrences may allow transmission of *M. agassizii*. Tortoises with slight nasal discharge, virtually undetectable, can also aerosolize bacteria by sneezing.

Although long-term studies on the effect of URTD on survival of individuals and populations have not been conducted, the evidence from surveys of desert tortoise populations and from Sanibel Island indicate that the disease can have severe negative impacts on population viability. Declines of 25-50% over 1-3 yr, and of 30-90% over 10 yr, are catastrophic for species that take 10-20 yr to reach maturity and have recruitment rates of 1-2%. Without marked improvement in recruitment rates, affected populations are unlikely to recover within a reasonable time frame.

Developing Questions and Conducting Surveys or Monitoring Programs

Third, questions related to management goals, and taking into account test interpretation and tortoise behavior, must be developed. Some of the most common

questions are: 1) What is the prevalence of antibodies against *M. agassizii* in the population of interest? 2) What is the distribution of seropositive tortoises relative to gender, age, habitat type, geography, or land use patterns? 3) Has the seroprevalence changed over time? 4) Is clinical disease present on the sites? 5) Is *M. agassizii* detectable from any tortoises? 6) Is there evidence, either from demographic profiles or tortoise remains, of large die-offs, or gaps in recruitment?

Once the goals have been established and questions developed, surveys or monitoring programs can be designed to collect the necessary samples and data. After field work has been conducted, samples collected and analysed, and test results analysed, the information can be used to develop management programs or research questions.

When trapping tortoises for studies or relocation, it is important to note any clinical signs that are present. Simply being aware of the possibility that URTD exists on a site may make field personnel more likely to detect clinical cases. While this does not guarantee that every case of URTD will be caught, it could give an early warning of potential problems, prevent sick tortoises from being put in holding pens with healthy ones, or transmitting the agent on equipment. It is easy to spray equipment with a mild bleach solution, or wipe down calipers and other equipment with alcohol soaked gauze. It is more difficult, and expensive, to construct separate holding facilities for healthy and sick tortoises, but alternatives are available. Ill tortoises can be kept in containers for a few days, long enough for monitoring and until test results come back.

Weighing Management Options and Formulating Management Plans

Knowing that URTD exists or has existed on a site, and the distribution of seropositive tortoises, may suggest management strategies to cope with potential problems. If tortoises testing positive are concentrated in one area, there may be opportunities to improve habitat so tortoises are not as stressed in times of food shortage, drought, or other adverse conditions. More monitoring time could be allocated to such areas in order to get an early warning in the case of a disease outbreak. If the site is close to or at carrying capacity, the population could be reduced so the remaining tortoises are under less intraspecific stress. Eggs could be collected from that site to use in headstart programs to ensure the genes are not lost in case of an outbreak.

If there is a population of tortoises into which relocated ones will be released, and the goal is to maintain the status quo relative to disease agents, then both populations need to be tested. If the recipient population and the donor population have approximately the same level of seropositive tortoises, and the site is well below carrying capacity, then it is probably acceptable to release seropositive tortoises, as long as the overall rate is not increased, and no tortoises actively shedding bacteria are released.

Releasing clinically ill tortoises, regardless of ELISA results, is not recommended. If a tortoise has a nasal discharge, palpebral edema, and conjunctivitis that persist for more than 24 hr, there is a substantial risk that the tortoise is shedding mycoplasma. Tortoises may shed more bacteria early in the infection, before they have developed a strong antibody response. Due to the secondary immune responses (Chapter 4), seropositive

tortoises that may have cleared the bacteria would be at risk of developing severe disease when exposed to a clinically ill tortoise, decreasing their survival probability.

If the goal is to establish, create, or maintain a URTD-free population, the question remains of what to do with seropositive, clinically healthy, culture and PCR negative individuals. If there is no secure place to hold the animals or a site where they can be released, then the most acceptable choice may be euthanasia. Euthanasia may save the animals from a long slow death, and prevents the chance of exposing negative animals. Alternatively, intensive monitoring of the population, with special attention paid to seropositive tortoises and samples collected several times per year, may allow rapid detection and removal of ill animals. However, such careful monitoring is beyond the capacity of most management agencies, and annual or biennial sampling may be all that can be accomplished.

Researchers sometimes need seropositive animals for projects. If one can be found, it is possible that animals could be donated. However, there are many issues to consider with that approach, mostly for the researcher. Researchers usually cannot accept animals that are made available suddenly, particularly for studies that require animals to be kept in laboratory settings. Costs, objectives, confounding factors of length of disease, genetics and site of origin, are all issues that must be addressed. It is expensive to keep tortoises in captive research settings, and safeguards must be in place to prevent their escape or theft. Research can be a politically and socially sensitive subject due to potentials for pain, illness, and death of animals, and nearly all animals are euthanatised at the end of the studies.

If the facilities and personnel are available, and the genetic material represented by the sick or seropositive tortoises is important, then a captive breeding program may provide the best solution. Clinically ill animals or those that test positive by culture or PCR should be maintained separately from clinically healthy, culture and PCR negative animals. Eggs can be collected from the females by induction of oviposition or at natural laying, or nests can be dug up. Eggs should not be allowed to hatch and hatchlings emerge from nests in pens inhabited by ill adults. Hatchlings can contract URTD from adults, and they generally become very ill very quickly. Eggs can be artificially or naturally incubated, and hatchlings released immediately or headstarted.

Summary of Conservation and Management Implications

In summary, the following points must be considered when making conservation and management decisions relative to URTD in gopher tortoises:

1) Goals must be clearly established.
2) Personnel must have appropriate training to recognise URTD, collect necessary samples, and interpret results.
3) Clear questions must be formulated.
4) Survey and monitoring programs must be developed and implemented, and precautions taken to ensure detection and prevent spread of URTD.
5) Management options must be weighed, and plans formulated and implemented that are consistent with established goals.
    a) Habitat manipulations.
    b) Relocate tortoises and maintain status quo relative to URTD.

c) Establish, create, or maintain URTD-free populations by careful monitoring, testing, and removal of infected tortoises.

d) Donate animals to research projects.

e) Establish captive breeding programs.

f) Euthanasia.

Further Research

It is not known how long *Mycoplasma agassizii* has been in gopher and desert tortoises, or if it occurs naturally in tortoises in other parts of the world. At least one other mycoplasma that causes URTD has been found in some populations of desert tortoises, and there may be other etiologic agents. An iridovirus was found in an ill gopher tortoise from Sanibel Island, and may have been the cause of the disease in that animal (Westhouse et al. 1996). Further research needs to be conducted on that agent.

The long-term effects of *M. agassizii* infection need to be studied. There are indications that liver function might be affected (Chapter 3), and that reproductive physiology is altered (Rostal et al. 1996). The impacts of altered basking and foraging behavior need to be quantified, as do the rates of transmission within natural populations. The role of maternal antibodies on the course of disease in hatchlings and juveniles is not understood. In order to fully understand the impacts of URTD on tortoise populations, many ares of research need to addressed, surveys undertaken, and long-term monitoring programs established.

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BIOGRAPHICAL SKETCH

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I spent most of my life on the West Coast of North America, and attended Humboldt State University, Arcata, California, receiving a Bachelor of Arts degree in Zoology, in spite of spending all my time in the Wildlife Department. I then ran an organic farm for several years before moving to Ames, Iowa, to attend Iowa State University. I studied gopher tortoises on Sanibel Island, Florida, en route to a Master of Science degree in Animal Ecology. Before defending my thesis in November 1990, I began my doctoral studies at University of Florida. I originally studied parasites of bobcats and Florida panthers, particularly their hookworms. However, I accepted a job with the gopher tortoise upper respiratory disease project, and switched dissertation research projects. I hope to move west and/or north soon after completion of my work on the project.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Donald J. Forrester, Chair
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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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